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13. ABSTRACT (Maximum 200 Words)

The BCCOE investigators made much progress in this second year. We demonstrated that the tumors induced in the ACI rat mammary gland regress when the implanted estradiol is removed. We showed that estrogen metabolites and glutathione conjugates in human nipple aspirates can be analyzed by ultra-performance liquid chromatograph/tandem mass spectrometry. Treatment of MCF-10F cells (ER negative) with estradiol or 4-hydroxyestradiol leads to their transformation, which encompases loss of heterozygosity in chromosome 13q12.3 and mutations in the TP53 tumor suppressor gene in chromosome 17p13.1. These changes occur even with the antiestrogen ICI1182780 present. We demonstrated that mutations are induced in a reporter gene in Big Blue rat mammary tissue by implantation with estradiol plus 4-hydroxyestradiol. We demonstrated that ovariectomized ERKO/Wnt-1 mice implanted with estradiol develop mammary tumors, even when the mice are also treated with the pure antiestrogen fulvestrant. We have characterized MCF-10F cell transformation at the combined levels of the complete genome and individual genes. We have analyzed estrogen metabolites, conjugates and depurinating DNA adducts in human nipple aspirates, MCF-10F cells and ERKO/Wnt-1 mouse mammary tissue. Development of a web-based model reference tool has progressed. Overall, we made great progress in defining the role of estrogen metabolism in the etiology of breast cancer and identifying potential biomarkers of susceptibility.

14. SUBJECT TERMS
Estrogens, estrogen-initiated cancer, depurinating estrogen-DNA adducts, analsysis of estrogen metabolites and conjugates, estrogen-induced mutation and cell transformation, overexpression of aromatase, gene expression by DNA icroarray, human breast ductal lavage, breast cancer prevention, biomarkers for breast cancer susceptibility

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SPECIFIC AIM 1 – CAVALIERI

A. Introduction

The basic hypothesis guiding this research is that endogenous estrogens can be oxidatively metabolized to catechol estrogen quinones that react with DNA to form specific DNA adducts to generate tumor-initiating mutations. The results obtained in animal models, cell culture and human breast tissue led us to select several compounds to prevent the genotoxicity of estrogens that we think is at the origin of breast cancer. The selected compounds target different steps involved in the mechanism of tumor initiation. Prevention studies will demonstrate that estrogen genotoxicity plays a critical role in the initiation of breast cancer. In addition, the results will lay the groundwork for designing a clinical research study of breast cancer prevention and developing bioassays for susceptibility to this disease. With these goals in mind, we are searching for an animal model for breast cancer.

B. Body

B-i. Methods and Procedures

In Specific Aim 1a, 8-10 week-old female rats were treated with 5 mg of E_2, 5 mg of 4-OHE_2 or 5 mg E_2 + 5 mg of 4-OHE_2 by implantation in silastic tubing in the scapular region of the back [1]. The rats were observed for 37-40 weeks. The silastic implant was removed from the rats that developed tumors to determine whether the tumors regressed.

In Specific Aim 1b, samples of human nipple aspirate fluid (obtained under UNMC exempt protocol #312-04-EX) were analyzed by ultraperformance liquid chromatography (UPLC) with detection by tandem mass spectrometry (MS/MS) to determine the presence of estrogen metabolites, estrogen-glutathione (GSH) conjugates and depurinating estrogen-DNA adducts in the breast fluid.

B-ii. Results

Specific aim #1a: Investigate the prevention of estradiol (E_2)-induced tumors in the mammary gland of female ACI rats by analyzing the profiles of estrogen mtabolites, conjugates and depurinating DNA adducts in treated animals at various time-points and the development of tumors in the animals.

Groups of 10 female ACI rats (susceptible to E_2-induced mammary tumors [1]) and 10 female Sprague-Dawley rats (refractory to E_2-induced mammary tumors) were treated with silastic implants of E_2, 4-OHE_2 or E_2 + 4-OHE_2 (Table 1). ACI rats treated with E_2 or E_2 + 4-OHE_2 developed mammary tumors (Table 1). However, when the implants containing the hormone were removed from some of the ACI rats after 27 or 28 experimental weeks, all of the tumors regressed and disappeared within 10 weeks (Table 2). The Sprague-Dawley rats did not develop tumors (Table 1). Some of the ACI and Sprague-Dawley rats initially treated with 4-OHE_2 were implanted with E_2 after 29 experimental weeks to see whether cells possibly initiated by 4-OHE_2 could be rapidly promoted to tumors by additional implantation of E_2 (Table 3). The only animals that developed tumors were the ACI rats. Thus, these animals do not provide a model for estrogen-initiated tumors because what is observed is reversible hyperplasia.

Table 1. Mammary Tumors in Female Rats Implanted with E_2 and 4-OHE_2[1]

Implanted Compound	Rat Strain	Effective No. of Animals	No. of DCIS[2] Tumor-Bearing Animals	% Tumor-Bearing Animals	No. of DCIS per rat	Week of Tumor Latency (avg.)
E_2	ACI	5[3]	57/52a	100	11.4	24
4-OHE_2	ACI	5[4]	0	0		
4-OHE_2 + E_2	ACI	6[3]	41/62a	100	6.8	26
Untreated	ACI	10	0	0		
E_2	SD	10	03a,10b			
4-OHE_2	SD	4[4]	0	0		
4-OHE_2 + E_2	SD	9	09b,1c			
Untreated	SD	8	0	0		

[1]Female ACI and Sprague Dawley rats were implanted s.c. with silastic tubules containing 5 mg of E_2, 4-OHE_2 or 5 mg each of E_2 and 4-OHE_2. The development of mammary tumors was monitored by weekly palpations from 16 weeks to the sacrifice of the animals upon becoming moribund. The experiment was terminated between 26 and 53 experimental weeks.

[2]The mammary glands were sectioned for histology and examined by Dr. T. Seemayer. All malignant mammary tumors were diagnosed as Ductal Carcinoma In Situ (DCIS). Other pathological findings seen in the mammary glands are as follows:

a. Number of animals with foci of benign tumor growth.
b. Number of animals with pronounced cystic change of the mammary glands and/or ductal ectasia.
c. Number of animals with benign fibroadenomas.

[3]Rats were removed for the study reported in Table 2.

[4]Rats were removed for the study reported in Table 3.

Table 2. Regression of Rat Mammary Tumors[1]

			Removal of Implants		Sacrifice[2]	
Implanted Compound - Removed	Rat Strain	Effective No. of Animals	Exp. Week (avg.)	No. of Palpable Tumors No. of Tumor-Bearing Animals	Exp. Week (avg.)	No. of Palpable Tumors No. of Tumor-Bearing Animals
E_2	ACI	4	28	15/4	37	0/0
4-OHE_2 + E_2	ACI	2	27	7/2	37	0/0

[1]E_2 and E_2 + 4-OHE_2 implanted ACI rats were selected and the implant was removed to study the regression of the palpable tumors. Upon sacrifice the original mammary tumor sites were examined, sectioned for histology and examined by Dr. T. Seemayer.

[2]All of the original mammary tumors sites were diagnosed as regressed tumors.

Table 3. Mammary Tumors in Female Rats first Implanted with 4-OHE_2 then with E_2[1]

First Implanted Compound (+ Second Implant)	Rat Strain	Effective No. of Animals	No. of DCIS No. of Tumor-Bearing Animals	% DCIS-Bearing Animals
4-OHE_2 (+E_2)	ACI	4	11/2[2]	50
4-OHE_2 (+E_2)	SD	5	0[3]	0

[1]Half of the 4-OHE_2-implanted ACI and SD rats were separated and each rat received an additional implant of 5 mg of E_2 at 29 experimental weeks. The rats were sacrificed when moribund, an average of 51 experimental weeks (ACI rats) and 53 experimental weeks (SD rats). The mammary glands were excised, sectioned for histology and examined by a pathologist.
[2]All of the mammary tumors examined from the ACI rats were diagnosed as DCIS.
[3]No DCIS tumors were diagnosed in the SD rats, a tumor from one rat was a benign fibroadenoma.

Specific Aim #1b: Analyze the profiles of estrogen metabolites, conjugates and depurinating DNA adducts in ductal lavage samples from women with and without breast cancer.

We have begun analyzing nipple aspirate samples from women without breast cancer (Figs. 1 and 2). Initially, we were using capillary HPLC to separate the estrogen metabolites, estrogen-GSH conjugates and estrogen-DNA adducts (Fig. 1). We now separate these compounds by ultraperformance liquid chromatography (UPLC), which has much more resolving power, with detection by MS/MS (Fig. 2).

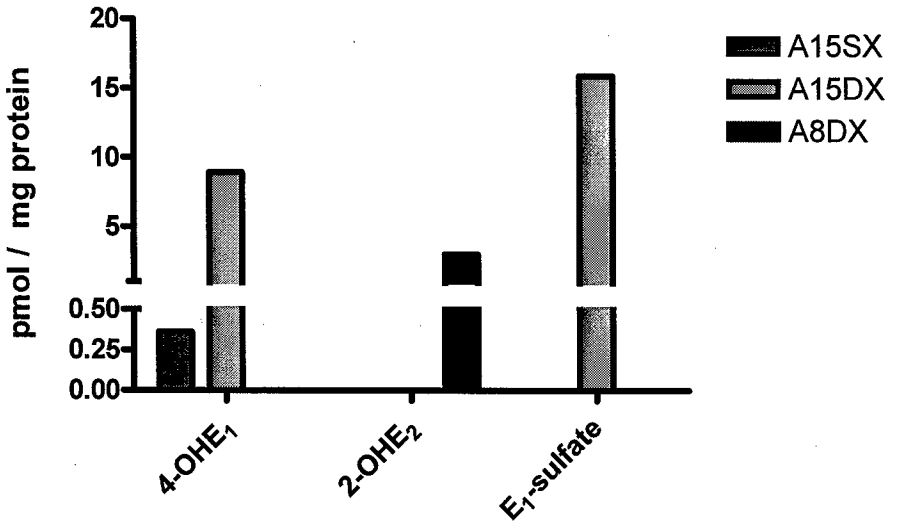

Figure 1. Initial analysis of nipple aspirate fluid by HPLC with MS/MS detection.

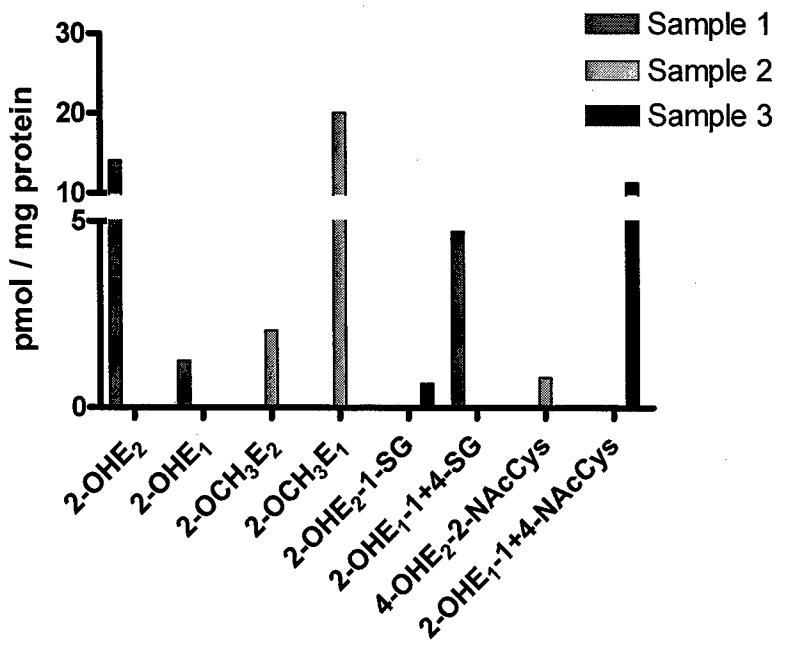

Figure 2. Analysis of nipple aspirate fluid by UPLC with MS/MS detection.

Almost all of the estrogen compounds detected in the samples analyzed by UPLC/MS/MS contained 2-OHE_2 (Fig. 2). This is not surprising, since these samples came from women without breast cancer, who can be expected to have lower levels of 4-OHE_2 [2]. These samples contained not only catechol estrogens and methylated catechol estrogens, but also GSH conjugates and their breakdown conjugates. WE will need to analyze many samples to see the variability and to identify biomarkers of susceptibility.

B-iii. <u>Proposed Research for the Next Year</u>

1. Specific Aim #1a: We have just begun a study in newborn Sprague-Dawley rats to determine whether they might provide a model for estrogen-initiated mammary tumors. Newborn Sprague-Dawley rats were injected daily on days 1-5 of life with 10 • g of E_2, 4-OHE_2 or 2-OHE_2 in 10 • Ł of trioctanoin:DMSO (95:5)/treatment [3]. At three weeks of age, the rats will be separated by sex and both the males and females will be maintained for approximately 12 months (or less if a health problem arises) to determine whether mammary or other tumors arise in the females and any tumors arise in the males. We hope that the treatment at an early age will result in tumor induction, and that we can develop a rat model for estradiol-initiated carcinogenesis.

Similarly, we will soon begin a study in newborn CD-1 mice, which will be injected on days 1-5 of life with 2 • g of E_2, 4-OHE_2 or 2-OHE_2 in 5 • Ł of trioctanoin:DMSO (95:5) per treatment. Since this is an established model, in which uterine adenocarcinomas are induced by E_2 and 4-OHE_2, and to a much lesser extent by 2-OHE_2 [3], we will test chemoprevention of these tumors by *N*-acetylcysteine (NAcCys) at high and low doses. The dams will be fed NAcCys in the diet starting one week before delivery of the pups and continuing through weaning. Once weaned, the pups will continue to receive the same diet as the dam. At 21 days of age, the males and females will be separated, and the males and dams will be sacrificed. The

females will be maintained for 12 months (or less, if health problems arise), sacrificed, and examined for tumors, especially uterine adenocarcinomas. If ingestion of NAcCys reduces the incidence of uterine adenocarcinomas, these results will indicate that metabolism of estrogens plays a role in the initiation of these tumors. Although we still hope to develop an animal model for estrogen-initiated mammary tumors, we may find that this is our best model for estrogen-initiated tumors.

2. Specific aim #1b: We plan to collect nipple aspirate samples from women with and without breast cancer, now that the protocol has received approval. The samples will be analyzed by UPLC with MS/MS detection. We will devote a considerable amount of time and attention to optimizing sample preparation and analysis to achieve the most sensitive, reproducible results possible. We hope that the limit of detection with the UPLC/MS/MS will be sufficient for analyzing metabolites, GSH conjugates or depurinating DNA adducts that can serve as biomarkers for susceptibility to breast cancer long before tumors are detected.

C. Key Research Accomplishments

1. We have demonstrated that all of the mammary tumors induced in female ACI rats by treatment with implants containing E_2 regress in a short period of time after the implant is removed.
2. We have concluded that the ACI rat mammary gland is not a model for estrogen-initiated mammary tumors because the E_2 is only promoting mutations already present in the mammary cells and the tumors regress upon removal of the E_2.
3. We have demonstrated that treatment of adult female ACI or Sprague-Dawley rats with 4-OHE_2 does not induce mammary tumors.
4. We have demonstrated that nipple aspirate fluid contains estrogen metabolites and GSH conjugates at levels of 1-20 pmol/mg protein. These potential biomarkers in a few microliters of sample can be separated by UPLC and detected by MS/MS.
5. We have demonstrated that collecting breast fluid by the non-invasive nipple aspiration technique is more successful for the study of estrogen biomarkers than collecting the fluid by ductal lavage, which is an invasive technique and results in more than a thousand-fold dilution of the fluid.

D. Reportable Outcomes

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Rogan, E.G. and Cavalieri, E.L. Estrogen metabolites, conjugates and DNA adducts: Possible biomarkers for risk of breast, prostate and other human cancers, In: Adv. Clin. Chem., (G.S. Makowski, G. Nowacki & K.-J. Hsiaom Eds.), 38:135-149, 2004.

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Cavalieri, E.L. and Rogan, E.G. The etiology of breast cancer. Prevention is now the solution. Era of Hope, Department of Defense Breast Cancer Research Program Meeting, Philadelphia, Pennsylvania, June 8-11, 2005.

Rogan, E.G. and Cavalieri, E.L. The role of estrogen metabolism in the initiation of human breast cancer. Era of Hope, Department of Defense Breast Cancer Research Program Meeting, Philadelphia, Pennsylvania, June 8-11, 2005.

Cavalieri, E.L., Saeed, M., and Rogan, E.G. The mechanism of tumor initiation by the human carcinogen diethylstilbestrol is similar to that of natural estrogens. Era of Hope, Department of Defense Breast Cancer Research Program Meeting, Philadelphia, Pennsylvania, June 8-11, 2005.

E. Conclusions

1. The mammary tumors induced by E_2 in ACI rats arise by promotion of already initiated cells, but these tumors regress upon withdrawal of E_2. Thus, the ACI rat mammary gland is not a good model for estrogen-initiated mammary tumors.

2. We are continuing to search for an animal model for estrogen-initiated mammary tumors.

3. Collection of human breast fluid by ductal lavage has several drawbacks in our experiments, most notably the large dilution in the lavage fluid. Collection by nipple aspiration is more successful and provides breast fluid that can be analyzed by LC/MS/MS. Ultra-performance liquid chromatography (UPLC) enables identification of about 30 estrogen metabolites, GSH conjugates and depurinating DNA adducts. We have detected metabolites and GSH conjugates in our initial studies of nipple aspirate fluid.

F. References

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2. Rogan, E.G., Badawi, A. F., Devanesan, P.D., Meza, J.L., Edney, J.A., West, W.W., Higginbotham, S.M., and Cavalieri, E.L. Relative imbalances in estrogen metabolism and conjugation in breast tissue of women with carcinoma: Potential biomarkers of susceptibility to cancer. *Carcinogenesis*, 24: 697-702, 2003.

3. Newbold, R.R., and Liehr, J.G. Induction of uterine adenocarcinoma in CD-1 mice by catechol estrogens. *Cancer Res.*, 60: 235-237, 2000.

SPECIFIC AIM 2 – RUSSO

A. Introduction

Breast cancer (BC) is the most commonly occurring neoplasm and the second most frequent cause of cancer death among women in the United States. There is a substantial amount of epidemiological, clinical and experimental evidence pointing to estrogens, e.g. 17β-estradiol (E_2), as being one of the most important etiological factors for the development and progression of BC (1-8). While the precise molecular mechanisms by which E_2 induces breast cancer have not been completely understood, the estrogen implication in breast cancer has been associated mostly with the estrogen receptor (ER) that mediates cell proliferation (7,8). The direct role of estrogen as tumor initiator has been supported by evidence that estradiol, catechol estrogens (CE), and their quinone derivatives may be genotoxic *in vitro* and *in vivo*, leading to various types of DNA damage, including single strand breaks and stable or depurinating DNA adducts (9-11). The 2-OHE_2 and 4-OHE_2 are two catechol metabolites of estrogen that could be oxidized to CE quinones (CE-Q) that may react with DNA. In particular, the 4-CE is oxidized to CE-3,4-Q which react with DNA to form depurinating adducts that generate apurinic sites that may lead to mutations in DNA (12-14).

To fully demonstrate that estrogens are carcinogenic in the human breast through one or more of the mechanisms explained above will require an experimental system in which, estrogen by itself or one of the metabolites would induce transformation phenotypes indicative of neoplasia in human breast epithelial cells (HBEC) *in vitro* and induce genomic alterations similar to those observed in spontaneous malignancies, such as DNA amplification and loss of genetic material that may represent tumor suppressor genes (17-31). We have shown experimental evidence that demonstrates that E_2 and its catechol metabolites, namely 4-hydroxyestradiol (4-OHE_2) and 2-hydroxyestradiol (2-OHE_2), are capable of *in vitro* transforming a HBEC, MCF-10F cell line, that is estrogen receptor alpha (ER-) negative and (ER+) positive (7,8). The E_2-mediated phenotypes and genomic changes were not abrogated by the pure antiestrogen ICI182780 (7,8) suggesting that in this model the ERα mediated pathway is not involved in the neoplastic process.

Due to the critical importance of determining if the transformation phenotypes are associated with specific changes at the genomic level, we have concentrated our main efforts during this grant period to identify the genomic changes in HBEC treated with E_2 and its metabolites as detailed below.

B. Body

B-i. Methods and Procedures

B-i-a. Cell lines

In the present work we have utilized MCF-10F cells (17,23) that have been treated twice a week during two weeks with E_2, 2-OHE_2 or 4-OHE_2 at the doses 0.007 nM and 70nM in the presence or absence of an equal concentration of ICI182780 (7,8). MCF-10F cells have been maintained in culture in DMEM: F-12 containing 5% horse serum and used at the following passages: MCF10-F (passage 127), cell treated with 0.007 nM E_2 (passage 6), 70 nM E_2 (passage 14), 0.007 nM 2-OHE_2 (passage 6), 70 nM 2-OHE_2 (passage 6), 0.007 nM 4-OHE_2 (passage 2), 70 nM 4-OHE_2 (passage 12), 0.007 nM E_2 +ICI (passage 3), 70 nM E_2 +ICI (passage 17), 0.007

nM 4-OHE_2+ICI (passage 3) and 70 nM 4-OHE_2+ICI (passage 9). MCF-10F cells treated with 0.007 nM or 1μg/ml benzo[*a*]pyrene (BP) (passage 12) were used as positive control for transformation.

B-i-b. Microsatellite analysis

DNA was extracted from the cells in culture when they were 70-80% confluent. The cells were treated with lysis buffer (100 mM NaCl, 20 mM Tris-Cl pH 8.0, 25 mM EDTA pH 8.0, 0.5% SDS) and 200μg/ml proteinase K was added and incubated at 65°C, 15 minutes with gentle agitation. The samples were cooled down on ice and treated with 100μg/ml RNase at 37 °C for 30 minutes. The DNA was purified with a phenol extraction (pH = 8.0) followed by chloroform: isoamyl alcohol (24:1). The aqueous layer was adjusted to 0.75M with ammonium acetate and the DNA was precipitated with 100% ethanol. The samples were centrifuged, dried and dissolved in distilled water. For microsatellite analysis, PCR was carried out in a final volume of 10 μl containing 1X PCR buffer (Invitrogen), 1.5 mM $MgCl_2$, 0.5 pmol of each primer, 100μM dNTPs, 0.25U TaqPlatinum (Invitrogen) and 60-90ng DNA. In each PCR reaction we have used fluorescent-labeled forward primer (Proligo, CA). The PCR conditions consisted of a denaturation step (5min at 94 °C) followed by 8 cycles at 94 °C for 20 sec, 60 °C for 45 sec (decreasing 0.5 °C per cycle) and 72 °C for 30 sec; 34 cycles at 94 °C, 20 sec, 56 °C for 45 sec and 72 °C for 30 sec. The fluorescent PCR was mixed with an internal standard size marker and fractionated using CEQ8000 (Beckman Coulter). For chromosome 13 and 17 analysis, 24 and 25 markers were used respectively (Tables I and II). A panel of five markers recommended to study MSI was also included: BAT25 (4q13; introns 16 of the c-kit oncogene), BAT26 (2p22-p21; fifth intron of MSH2 gene); D2S123 (2p16; MSH2 gene); D5S346 (5q22; APC gene) and with D17S250 (17q22). Loss of heterozygosity (LOH) was defined as a total loss (complete deletion) or a 30% or more reduction in the signal of one of the heterozygous alleles compared with the control MCF-10F DNA (24). Each reaction was repeated at least three times for each DNA.

B-i-c. Cloning and sequencing of 290 bp of TP53 exon 4

Non-fluorescent TP53-Dint primers were used to amplify 291bp and 296bp fragments of TP53 exon 4 (Table 2) (25). The following conditions were used to amplified: denaturalization at 94 °C for 2 min, 35 cycles of: 94 °C for 30sec, 55 °C for 45 sec and 72 °C for 45 sec, and a final extension at 72 °C for 10 min. The Expand High Fidelity Taq (Roche) was used in the amplification and the PCR products were cloned in TOPO TA vector (Invitrogen). DNAs from transformed MCF-10F with 70nM $4OHE_2$ and DNA from MCF-10F without treatments were used as templates. Several white colonies were picked-up and plasmids were isolated using standard methods. The cloned fragments were sequenced at the Fox Chase Cancer Center Sequencing Facility.

B-ii. <u>Results</u>

Genomic alterations in chromosome 13 and chromosome 17 are known to play an important role in the initiation and progression of human breast cancer (18-20). Chromosome 13 contains the breast cancer susceptibility gene BRCA2 located in chromosome 13q12-q13 (22). Chromosome 17 contains the tumor suppressor gene TP53 located at 17p13.1 and BRCA1 at 17q21.31 (21).

B-ii-a. Microsatellite analysis

We have found that using the microsatellite marker D13S893 located at 13q12.3 the parental cell line MCF-10F shows two peaks, one at 185bp and the other at 189bp, representing two alleles (Figure 1). The cells treated with 0.007nM E_2 or 70nM E_2 show loss of heterozygosity (LOH) with complete loss of one allele; only one peak at 185bp was detected and the other peak at 189bp was lost and this effect was not prevented by ICI (Figure 1). The treatment with both doses of 4-OHE_2 produced the complete loss of one allele; only one peak at 185bp was detected and the other peak at 189bp was lost and this effect was not prevented by the antiestrogen ICI (Figure 1). MCF-10F treated with 2-OHE_2 at the doses 0.007nM and 70nM show two peaks as the control MCF-10F without treatment (Figure 1). The treatment with 2-OHE_2 produced a similar effect as E_2 and 4-OHE_2 (total loss of one peak) only at a higher dose (3.6μM) (Figure 1). The cells treated with BP show two peaks with the marker D13S893 as MCF-10F cells without treatment (Figure 1). We have not found microsatellites changes with any of the other 23 markers used for chromosome 13 (Table 1).

Table 1. Microsatellite markers used for chromosome 13 studies. The sequence of the primers, the marker location, size of the PCR product and repeat type are indicated in each case.

Marker	Primer Reverse (5' → 3')	Primer Forward (5' → 3')	Location	Allele size range (bp)	Repeat Type
D13S1244	CTGTTTATGGCTGAGAAGTATGC	TCAACAAGTGGATTAAGAAACTGTG	13q12.2	86-108	dinucl.
D13S1242	GCCCCCAGTCAGGTTT	GTGCCCAGCCAGATTC	13q12.3	198-248	dinucl.
D13S1246	AGTGGCTATTCATTGCTACAGG	GAGCATGTGTGACTTTCATATTCAG	13q12.3	177-213	dinucl.
D13S290	CAAATTCCTCAATTGCAAAAT	CCTTAGGCCCCATAATCT	13q12.3	176-194	dinucl.
D13S893	TTGCTAATCTCCTTAAAAAATAACG	ATATTGCACAACTTTGTGTAGAGC	13q12.3	182-190	tetranucl
D13S260	CCAGATATAAGGACCTGGCT	TCAGATTGCTAAGCATGTACC	13q12.3	129	dinucl.
D13S171	ATTCTTCCTCCCATCTTTGCTCTC	AAAATTCCCCAAAAAACTACCTACC	13q12-13	239	dinucl.
D13S267	TCCCACCATAAGCACAAG	GGCCTGAAAGGTATCCTC	13q13.2	148-162	dinucl.
D13S1293	AAATAACAAGAAGTGACCTTCCTA	TGCAGGTGGGAGTCAA	13q13.1	119-139	dinucl.
D13S220	TGCATTCTTTAAGTCCATGTC	CCAACATCGGGAACTG	13q13.2	191-203	dinucl.
D13S219	GCATGAGGCAATTGGTTATA	GGAACAGCAAAGCAAATATG	13q13	156	dinucl.
D13S1253	CAGAGCCGTGGTAGTATATTTTT	CCTGCATTTGTGTACGTGT	13q14	120-144	dinucl.
D13S218	TCGGGCACTACGTTTATCTA	CCTGCAAACATATGGATGAT	13q12.2	213	dinucl.
D13S175	TGCATCACCTCACATAGGTTA	TATTGGATACTTGAATCTGCTG	13q11	101-113	dinucl.
D13S283	GCCATTCCAAGCGTGT	TCTCATATTCAATATTCTTACTGCA	13q12.1	128-155	dinucl.
D13S221	GAGATCGTGCAGCACTTGT	TAGCCATGATAGGAAATCAACC	13q12.1	223-243	dinucl.
D13S1244	CTGTTTATGGCTGAGAAGTATGC	TCAACAAGTGGATTAAGAAACTGTG	13q12.1	86-108	dinucl.
D13S289	TGCAGCCTGGATGACA	CTGGTTGAGCGGCATT	13q12.2	259-276	dinucl.
D13S263	CCCAGTCTTGGGTATGTTTTTA	CCTGGCCTGTTAGTTTTTATTGTTA	13q13	145-165	dinucl.
D13S328	GGGGGTTTACTTTTGTGAAG	GCCTGAACATCACTTTTTTG	13q14.3	128	dinucl.
D13S273	ATCTGTATGTCCTCCTTTCAATG	CTGNGGCAAAAACAACTCTT	13q14.3	235-259	dinucl.
D13S272	AGCTATTAAAGTTCCCTGGATAAAT	ATACAGACTTCCCAGTGGCT	13q14.3	131-143	dinucl.
D13S176	ATATTCAGACAAAAGCCAAGTTA	CTGTGGGATTCCTTAGTGATAC	13q14.3	211-227	dinucl.
D13S269	CAAAGTGGTTCATCTTGGTCT	TGTCTTCCAGCAGGGC	13q22	116-140	dinucl.

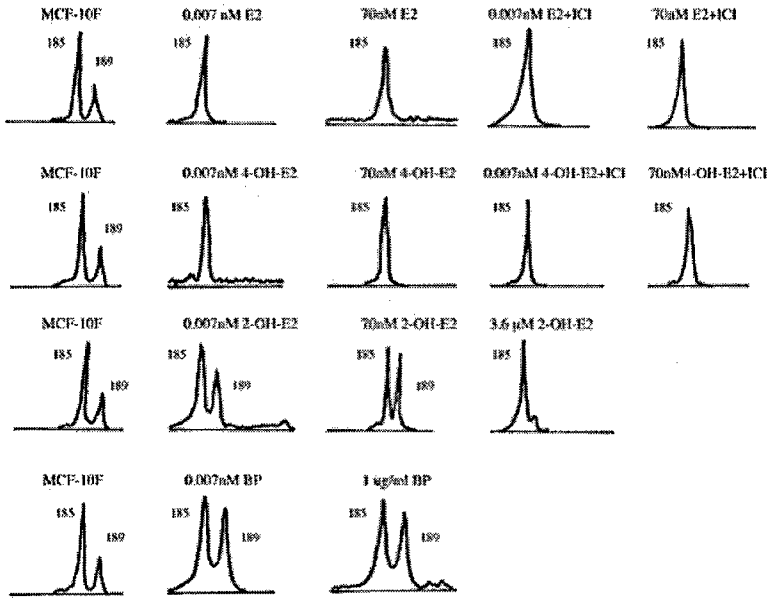

Figure 1. DNA from the E_2 or 4-OHE_2 transformed cells at two different doses: 0.007nM and 70nM alone or in combination with ICI182780 and DNA from 2-OHE_2 transformed cells at 0.007nM, 70nM or 3.6μM were utilized for performing the analysis. The parental cell line MCF-10F shows two peaks, one at 185bp and another at 189bp, representing two alleles. The cells treated with 4-OHE_2 or E_2 at all the doses tested produced LOH with complete loss of one allele; only one peak at 185bp was detected; the other peak at 189bp was lost and this effect was not prevented by ICI. The cells treated with 2-OHE_2 at the lower doses (0.007nM and 70nM) show two peaks although, at the highest dose (3.6μM) they show only one peak. Cells treated with 0.007nM BP and 1μg/ml BP were used as controls.

Table 2. Microsatellite markers used for chromosome 17 studies. The sequence of the primers, marker location, sizes of the PCR product and repeat type are indicated.

Marker	Primer Reverse (5' → 3')	Primer Forward (5' → 3')	Location	Allele size range (nt)	Repeat Type
D17S926	CCGCAGAAGGCTGTTGT	GCAGTGGGCCATCATCA	17p13.3	243-260	dinucl.
D17S1840	TGGGGCAGACTTGGTCCTT	GCCTGGGCGACAGAGTGA	17p13.3	173-225	dinucl
D17S1528	CAGAGGTGGAGATAAGGG	AGTAGCCAGGAGGTCAAG	17p13.3	188-226	dinucl.
D17S831	GCCAGACGGGACTTGAATTA	CGCCTTTCCTCATACTCCAG	17p13.3	224-246	dinucl
D17S1810	CCTAGTGAGGGCATGAAAC	TGTCCACTGTAACCCCTG	17p13.3	108-114	dinucl
D17S919	GCTTAATTTTCACGAGGTTCAG	AGGCACAGAGTGAGACTTG	17p13.3	148	tetranucl
D17S1832	TGTGTGACTGTTCAGCCTC	ACGCCTTGACATAGTTGC	17p13.3	179-195	dinucl
D17S260	CTCCCCAACATGCTTTCTCTC	AATGGCTCCAAAAGGAGATATTG	17p13.3	147	mononucl
D17S906	TTCTAGCAGAGTGAAACTGTCT	AGCAAGATTCTGTCAAAAGAG	17p13.3	335	tetranucl
D17S1149	CGCTGATCTGTCAGGCAGCCCT	AACAAGAGTGAACTCCATAGAGAG	17p13.3	276	tetranucl
D17S720	GAATTCTGAGCATATTGTTTGCCTG	CCAGCCTTGGCAACATAGCAAGA	17p13.3	221	tetranucl
D17S578	CTGGAGTTGAGACTAGCCT	CTATCAATAAGCATTGGCCT	17p13.3	134-174	dinucl
D17S960	TAGCGACTCTTCTGGCA	TGATGCATATACATGCGTG	17p13.3	127-135	dinucl
D17S1881	TAGGGCAGTCAGCCTTGTG	CCCAGTTTAAGGAGTTTGGC	17p13.3	216-230	dinucl
D17S1353	TACTATTCAGCCCGAGGTGC	CTGAGGCACGAGAATTGCAC	17p13.3	184-222	dinucl
D17S938	ATGCTGCCTCTCCCTACTTA	GGACAGAACATGGTTAAATAGC	17p13.2	145	dinucl
D17S796	AGTCCGATAATGCCAGGATG	CAATGGAACCAAATGTGGTC	17p13.2	144-174	dinucl
TP53-Pent	ACAAAACATCCCCTACCAAACAGC	ACTCCAGCCTGGGCAATAAGAGCT	17p13.1	125-155	pentanucl
TP53-Dint	GCAACTGACCGTGCAAGTCA	ATCTACAGTCCCCCTTGCC	17p13.1	291-296	dinucl

D17S250	GCTGGCCATATATATATTTAAACC	GGAAGAATCAAATAGACAAT	17q12	151	dinucl
THRA1	CGGGCAGCATAGCATTGCCT	CTGCGCTTTGCACTATTGGG	17q11.2-q12	158-176	dinucl
D17S855	ACACAGACTTGTCCTACTGCC	GGATGGCCTTTTAGAAAGTGG	17q21	145-150	dinucl
D17S800	ATAGACTGTGTACTGGGCATTGA	GGTCTCATCCATCAGGTTTT	17q21.1	168- 178	dinucl
D17S579	CAGTTTCATACCAAGTTCCT	AGTCCTGTAGACAAAACCTG	17q21.3	111-125	dinucl
GH	AGTCCTTTCTCCAGAGCAGGT	TCCAGCCTCGGAGACAGAAT	17q22-24	243	dinucl

For chromosome 17, we have found that using the marker TP53-Dint located in chromosome 17p13.1, exon 4 of the tumor suppressor gene TP53, the parental cell line MCF-10F has only one peak at 296bp (Figure 2), whereas the MCF-10F cells transformed with 0.007nM E_2 or 70nM E_2 have two peaks, 296bp and 291bp. The antiestrogen ICI182780 has not prevented the appearance of the shorter allele (Figure 2). MCF-10F cells treated with 4-OHE_2 alone or in combination with ICI182780, show two peaks, one at 296bp and another shorter, at 291bp (Figure 2). MCF-10F cells treated with 2-OHE_2 at the lowest concentrations (0.007nM and 70nM) show only one peak at 296bp as the parental cell line MCF-10F (Figure 2). The 2-OHE_2 shows two peaks (296bp and 291bp) only at the highest dose (3.6μM) (Figure 2). These data suggest that 4-OHE_2 and E_2 at the doses tested (0.007nM, 70nM) and 2-OHE_2 at a higher concentration (3.6μM) produced a deletion in TP53, exon 4 and this effect was not prevented by the antiestrogen ICI182780.

The cells treated with the carcinogen BP exhibit only one peak at 296bp as the control MCF-10F without treatment (Figure 2), showing that the mutation in this region is a specific effect of the E_2 and its metabolites.

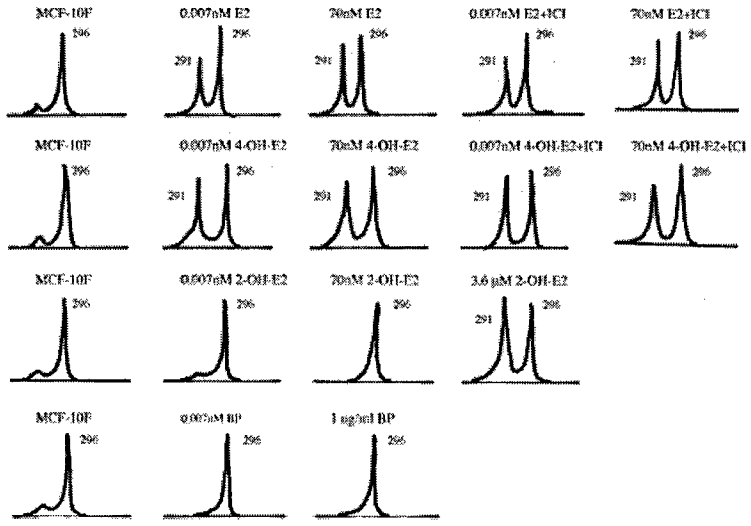

Fig. 2. DNA from the E_2 or 4-OHE_2 transformed cells at two different doses: 0.007nM and 70nM alone or in combination with ICI182780 and DNA from 2-OHE_2 transformed cells at 0.007nM, 70nM or 3.6μM were utilized for performing the analysis. Cells treated with 0.007nM BP and 1μg/ml BP were used as controls.

B-ii-b. Deletion in TP53, exon 4

To study in detail exon 4 of TP53, this region was amplified from MCF-10F transformed with 70nM 4-OH-E_2. DNA from MCF-10F without treatment was used as control. The primers TP53-Dint were used in the amplification and the PCR product obtained was of 290bp. The fragment was cloned and sequenced. The sequence alignment of TP53 exon 4 wild type and mutated is shown (Figure 3). Using DNA from MCF-10F treated with 70nM 4-OHE_2 as

template in the PCR reactions, three colonies out of eleven have a deletion in TP53 exon 4 (3/11); in the other colonies, TP53 exon 4 was wild type (8/11). The deletion consisted of 5bp, TCCCA, and it was between codons 98 and 100. TP53 exon 4 has 279 bases in length, from base 12021 until 12300, and encodes 93 aminoacids (aminoacid 33 until 123). TP53 protein has several domains: transactivation domain (aminoacids 1 to 50), SH3 binding domain (aminoacids 63 to 97), DNA binding domain (aminoacid 110 to 290), oligomerisation domain (326 to 356) and the negative regulator domain (360 to 394). The TP53 5bp deletion found between codons 98 and 100 would affect mainly the DNA binding domain and probably the others domains due to a different structure of the protein. All the clones isolated from MCF-10F without treatment harbored the wild type TP53 exon 4.

A)

12021
tccccttgccgtcccaagcaatggatgatttgatgctgtccccggacgatattgaacaatggctcactgaagacccaggtccagatgaagc
tcccagaatgccagaggctgctccccgcgtggcccctgcaccagcagctcctacaccggcggcccctgcaccagccccctcctggcccc
tgtcatcttctgtccc**ttccc**agaaaacctaccagggcagctacggtttccgtctgggcttcttgcattctgggacagccaagtctgtgacttgc
acgg 12300

B)

Wild type
33
S P L P S Q A M D D L M L S P D D I E Q W L T E D P G P D E A P R M P E A A P
R V A P A P A A P T P A A P A P A P S W P L S S S V P **S Q K T Y Q G S Y G F R**
L G F L H S G T A K S V T C T
125

Mutated
33
S P L P S Q A M D D L M L S P D D I E Q W L T E D P G P D E A P R M P E A A P
R V A P A P A A P T P A A P A P A P S W P L S S S V P **E N L P G Q L R F P S G**
L L A F W D S Q V C D L H
123

Fig. 3. TP53, exon 4 wild type and mutated. A) Nucleotide sequence from the wild type TP53 exon 4 (bases 12021-12300); the 5 bases missing by treatment with 70nM 4-OHE$_2$ are underlined. B) Amino acid composition of TP53 exon 4 from the wild type (amino acid 33-125) and mutated (amino acid 33-123); the amino acids that would be different between the wild type and mutated are underlined.

B-iii. Next Proposed Plan of Research

B-iii-a. To test if preventive agents are able to abrogate the transformation phenotypes and the genotoxic effect in Ch 13 and p53

Tamoxifen will be used to block the action of estrogens through the receptor-mediated mechanisms. D3T, a monofunctional inducer of phase 2 enzymes, and NAcCys ± vitamins C and E will also be used as preventive compounds. The estrogen compounds will be used in the physiologic range of 0.007 nM to 70 nM, at which E_2 and metabolites induced transformation of

HBEC. Dose-response curves for the preventive compounds will be established to determine the efficacious doses to be used. The cells will be treated for two periods of 24 h each. The first treatment will be initiated 24 h post-plating of MCF-10F cells. The second treatment will be administered after 6 days. Aliquots will be collected after each treatment and after the cells have been selected in agar methocel. The data obtained will provide a direct evidence of the importance of affecting the homeostatic pathway of estrogen metabolism by measuring the transformation phenotypes *in vitro* and the tumorigenic response in the heterologous host.

B-iii-b. Assays for anchorage-independent growth in agar methocel

Treated and control cells collected will be seeded at a concentration of $1x10^4$ cells in 0.8% methylcellulose in 24 multi-well plates pre-coated with a layer of 0.9% agar. The cells will be fed daily with fresh medium. All cultures will be evaluated 24 h post-plating for detection of cell aggregates that might bias the final results, and 21 days post-plating for determination of survival efficiency, colony number, colony efficiency, and colony size.

B-iii-c. Chemotaxis and invasiveness assays

Chemotaxis and invasiveness will be determined using Boyden-type Transwell chambers (Costar, Cambridge, MA) separated by a porous polycarbonate filter (8 mm pore size) (Nucleopore, Pleasanton, CA) coated with reconstituted basement membrane material (Matrigel, Collaborative Research, Bedford, MA). Trypsinized cells will be seeded in the upper chamber and fibronectin at 1.0 μg/mL will be placed in the lower chamber as chemoattractant. The total number of cells that cross the membrane during a 12-h period of incubation will be determined under a light microscope upon fixation of the filters and staining by Diff Quick (Sigma Chemical Co., St. Louis, MO).

B-iii-d.Tumorigenic assay in SCID mice

The tumorigenic capabilities of transformed HBEC treated with estrogen or its metabolites will be tested by their inoculation to 45-day-old female SCID mice which will be obtained from the Fox Chase Cancer Center Animal Care Facilities. Trypsinized cells ($1.0X10^7$) will be suspended in 0.1 mL phosphate buffered saline and injected into the mammary fat pad of SCID mice. Tumorigenesis in these animals will be determined as described previously [32,33]. Latency period and tumor growth rate will be used as parameters of tumorigenesis.

B-iii-e. Detection of genomic changes

The DNA and microsatellite analysis will be performed as indicated and described in section B-i-b and c.

C. Key Research Accomplishments

1. MCF-10F cells treated with all doses of E_2 and 4-OHE_2 and only with the highest dose of 2-OHE_2, either alone or in combination with the antiestrogen ICI, exhibited LOH in the region 13q12.3 when using the marker D13S893, which is at approximately 0.8cM upstream of the heritable breast cancer susceptibility gene BRCA2, with complete loss of one of the alleles in this region.

2. MCF-10F cells transformed with 0.007nM E_2 or 70nM E_2 have a mutation in TP53-Dint located in chromosome 17p13.1, exon 4 of the tumor suppressor gene TP53. The antiestrogen ICI182780 has not prevented the appearance of this mutation.

3. MCF-10F cells treated with either 2-OHE_2 or 4-OHE_2 alone or in combination with ICI182780, show a mutation in TP53-Dint located in chromosome 17p13.1, exon 4 of the tumor suppressor gene TP53.

4. The cells treated with the carcinogen BP do not show a mutation in TP53-Dint located in chromosome 17p13.1, exon 4 of the tumor suppressor gene TP 53, indicating that the mutation in this region is a specific effect of E_2 and its metabolites.

D. Reportable Outcomes

1. Fernandez, S.V., Russo, I.H. and Russo, J. Estrogen and its metabolites 4-hydroxy-estradiol and 2-hydroxy-estradiol induce mutation of p53 and LOH in a gene located 0.8cM of BCR2 in human breast epithelial cells. Proc. Am. Assoc. Cancer Res.46: 2098a, 2005.

2. Fernandez, S.V., Russo, I.H. and Russo, J. Estrogen and its metabolites-4-Hydroxy estradiol and 2-Hydroxyestradiol induce mutations in human breast epithelial cells. Proc. Era of Hope, Philadelphia, June 8-11, 2005.

E. Conclusions

During the second year of our award we have clearly demonstrated that estradiol (E_2), 4-OHE_2, and, to lesser extent 2-OHE_2 induced genotoxic effects in HBEC. MCF-10F cells treated with all doses of E_2 and 4-OHE_2 and only with the highest dose of 2-OHE_2, either alone or in combination with the antiestrogen ICI, exhibited LOH in the region 13q12.3 when using the marker D13S893, which is at approximately 0.8cM upstream of the heritable breast cancer susceptibility gene BRCA2, with complete loss of one of the alleles in this region. A mutation in TP53-Dint located in chromosome 17p13.1, exon 4 of the tumor suppressor gene TP 53 shows a high selectivity of the E_2 and 4-OHE_2, that is not achieved by other carcinogenic agents like BP and with lower doses of 2-OHE_2. Altogether, the failure of the antiestrogen to stop the genotoxic effects and the emergence of the transformation phenotypes support the concept that this effect is a non-receptor mediated process.

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SPECIFIC AIM 3 - GUTTENPLAN

A. Introduction

Epidemiological studies suggest that natural and synthetic estrogens have etiologic roles in breast cancer (1,2). Mechanistic studies indicate that estrogens could play two major roles in breast cancer. One of these roles is to induce estrogen receptor-positive mammary cells into proliferation (3). This mechanism is well studied. Recent research conducted in our group and in other laboratories indicates that estrogens play a second critical role in breast cancer (4,5). In this role, estrogens act as chemical carcinogens, inducing mutations to initiate breast cancer. It is possible that these two roles act in concert to induce breast cancer.

Breast estrogen is metabolized to form two different catechols [2-hydroxy E_1/E_2 and 4-hydroxy E_1/E_2] that are further metabolized to ortho-quinones [E_1/E_2-2,3-quinone (Q) and E_1/E_2-3,4-Q] [1], which then may react with DNA to form adducts. Two types of DNA adducts are formed: stable adducts (removed from DNA by excision repair) and depurinating adducts (spontaneously lost from DNA) (6). Oxidation of 2-OHE_1/E_2 results in the formation of E_1/E_2-2,3-Q, which form primarily stable adducts in DNA (7). On the other hand, oxidation of 4-OHE_1/E_2 results in the formation of E_1/E_2-3,4-Q, which form primarily depurinating DNA adducts (6). Since the quinone (E_1/E_2-3,4-Q) of the more potently carcinogenic 4-OHE_1/E_2 forms primarily depurinating adducts, we hypothesize that estrogen-induced depurinating adducts have a central role in the initiation of breast cancer. As depurinating adducts leave DNA, apurinic sites are left behind. Recently, we found that apurinic sites formed by E_2-3,4-Q in mouse skin induced mutations by error-prone DNA repair (6). This suggests that estrogen may induce mutations by apurinic site misrepair in an unknown cancer gene to initiate sporadic breast cancer.

B. Body

B-i. Methods and Procedures

Mutagenesis experiments were carried out using either Big Blue ™ Fisher rats or Big Blue ™ rat embryo cells in culture

1. Analysis of Mutations of lacI or CII Reporter Genes in the Mammary Gland and Liver Induced by Subcutaneous Implantation of E_2, 4-OHE_2 or (4-OHE_2+E_2) in Female Big Blue Rats

In this study rats were treated with implants of silastic tubing containing either E_2, 4-OHE_2 or (4-OHE_2+E_2) and sacrificed either 10 or 20 weeks later. Following euthanasia the mammary glands and liver from the animals were flash frozen with liquid N_2 and stored at -80 °C. Left and right inner and outer inguinal glands were taken and analyzed separately.

[1] Abbreviations: E_2, estradiol; 4-OHE_2, 4-hydroxyestradiol; 2-OHE_2, 2-hydroxyestradiol; E_2-3,4-Q, 3,4-E_2-quinone; E_2-2,3-Q, 2,3-E_2-quinone

Compound	Dose	Total	Sacrifice Dates
4-OHE_2	5 mg	14 rats	7 rats @ 10 wk 7 rats @ 20 wk
4-OHE_2 + E_2	5 mg + 5 mg)	13 rats	7 rats @ 10 wk 6 rats @ 20 wk
E_2	5 mg	10 rats	5 rats @ 10 wk 4 rats @ 20 wk

Additionally, 10 untreated rats were taken as controls.

2. Treatment of BB cells in culture

Cells were treated with either E_2-3,4-Q, E_2-2,3-Q or 4-OHE_2 at the indicated concentrations in confluent and subconfluent cultures. When subconfluent, plates were about 20% confluent. Cells were treated in DMEM without serum, overnight and washed and placed in DMEM + 10% FBS. Confluent plates were split to 20% and all were allowed to grow to confluence. At this time, the subconfluent group was split to 20% confluence and all plates were treated again under the same conditions as above. After selected numbers of treatments, a portion of the cells were saved for analysis and the rest were plated for additional treatments. After the final indicated number of treatments cells were harvested.

Mutagenesis

After cells or tissues were collected, DNA was isolated by a dialysis method and packaged into phages. Mutants were analyzed at the cII gene by selection at 25 °C in an *Hfl E coli* strain and total plaque forming units (pfu) were determined by plating in the same strain at 37 °C. The ratio of mutant/nonmutant plaques is the mutant fraction.

3. Sequencing

Mutant plaques were picked from the agar, resuspended in phage buffer and amplified by PCR. The amplified sequences were sent to the University of Victoria for sequencing.

B-ii. Results

Analysis of Mutations of lacI or CII Reporter Genes in the Mammary Gland and Liver Induced by Subcutaneous Implantation of E_2, 4-OHE_2 or (4-OHE_2+E_2) in Female Big Blue Rats

As seen in Table 1 there was little observable effect of the treatments in mammary or liver at 10 weeks (e.g., the mutant fractions in all of the groups were similar). However, at 20 weeks all of the treated groups exhibited a higher mutant fraction in mammary tissue than the control group (Table 2). Mutagenesis for the 20 week liver samples is not yet complete. The differences in mammary tissue were statistically significant for E_2 + 4-OHE_2 and E_2 groups vs. the control group and approached significance ($p = 0.06$) for the 4-OHE_2 group. This result is consistent with the hypothesis that estrogens lead to mutagenic metabolites.

Table 1. Mutant fractions in BB rat mammary and liver tissue after 10 weeks treatment with E_2 , 4-OHE_2 , E_2 + 4-OHE_2, and in tissue from untreated rats

treatment	# of rats	Tissue	MF	SD
E_2 + 4-OH	7	mammary	1.27	0.91
4-OHE_2	7	mammary	1.01	1.84
E_2	5	mammary	1.14	0.69
Control	4	mammary	0.94	0.61
E_2 + 4-OH	7	liver	1.60	0.83
4-OHE_2	7	liver	1.80	0.96
E_2	5	liver	1.30	0.60
Control	4	liver	1.40	0.57

[1]For mammary tissue the values represent the average of the MF's for left and right inner and outer inguinal glands.

Table 2. Mutant fractions in BB rat mammary and liver tissue after 20 weeks treatment with E_2, 4-OHE_2 , E_2 + 4-OHE_2, and in control rats

treatment	# of rats	tissue[1]	mean MF	SD
E_2 + 4-OHE_2	6	mammary	1.93*	0.32
4-OHE_2	8	mammary	1.65	0.69
E_2	4	mammary	2.06*	0.49
Control	6	mammary	0.95	0.50

[1]For mammary tissue the values represent the average of the MF's for left and right inner and outer inguinal glands.
*$p < 0.05$ vs. control

Sequencing

The sequences in a number of mutant plaques were compared. Important differences in the E_2 + 4-OHE_2 and the E_2-treated or control groups were noted (Table 3). The only group that had a significant percentage (36%) of mutations at A:T base pairs was the E_2 + 4-OHE_2 group. Results for the 4-OHE_2 alone group are not yet complete. These results are consistent with our hypothesis that the product of metabolism of 4-OHE_2 (E_2-3,4-Q) leads to depurination at A:T base pairs, and this is represents a major mutagenic lesion resulting from estrogens.

Table 3. Distribution of mutants from estrogen-treated and control BB rat mammary tissue

	GC:AT	GC:TA	GC:CG	AT:GC	AT:CG	AT:TA	ins/del[1]	other	mutants
E_2 + 4-OHE_2	8	2	3	2	2	5	3	0	25
E_2	6	5	1	0	0	1	2	0	15
control	4	1	1	0	0	0	2	0	8

[1]Insertions or deletions

Treatments of BB rat embryo cells with estrogen metabolites

Confluent or subconfluent BB cells were treated multiple times with estrogen metabolites. Preliminary experiments had showed that a single treatment did not lead to mutagenesis and therefore multiple treatments were employed. As different DNA repair systems may act in confluent and subconfluent cultures, both conditions were tested. The estrogen quinones are unstable at neutral pH, and therefore cells were treated at pH 6.0 where they are more stable. So as not to cause cell death, treatments were kept to one hour. Triplicate plates were assayed. Under these conditions no statistically significant increase over background levels of mutagenesis was observed in confluent or subconfluent cultures (Tables 4-6).

Table 4. Effects of estrogen metabolites in confluent cells on mutant fractions of BB rat cells: 3x treated

treatment		MF x 10^{-5}	Ave MF	SD
(3X treated)				
2,3 Qu 1 uM	1	5.22		
2,3 Qu	2	2.77		
2,3 Qu	3	4.27	4.08	1.23
3,4 Qu 6 uM	1	3.07		
3,4 Qu	2	4.90		
3,4 Qu	3	3.60	3.86	0.94
Control	1	1.66		
Control	2	3.05		
Control	3	3.94	2.88	1.15

Table 5. Effects of estrogen metabolites in confluent cells on mutant fractions of BB rat cells: 6x treated

treatment		MF x 10^{-5}	Ave MF	SD
(6X treated)				
2,3 Qu 1 uM	1			
2,3 Qu	2	1.67		
2,3 Qu	3	3.76	2.72	1.48
3,4 Qu 6 uM	1	3.18		
3,4 Qu	2	2.54		
3,4 Qu	3	2.35	2.69	0.44
Control	1	3.72		
Control	2	2.13		
Control	3	8.50	2.92	3.32

Table 6. Effects of estrogen metabolites in subconfluent cells on mutant fractions of BB rat cells: 11 x treated

treatment		MF x 10^{-5}	MF ave x 10^{-5}	SD
2,3 Qu 6 uM	11 x 1	5.22		
2,3 Qu	2	2.77		
2,3 Qu	3	4.27	4.08	1.23
3,4 Qu 1 uM	11 x 1	3.07		
3,4 Qu	2	4.90		
3,4 Qu	3	3.60	3.86	0.94
Control	11 x 1	1.66		
Control	2	3.05		
Control	3	3.94	2.88	1.15
BaP[1] 0.4 uM	1 X	11.90		
BaP 0.4 uM	1 X	13.60		
BaP 0.4 uM	1 X	10.60	12.03	1.50

[1]Benzo[*a*]pyrene (positive control).

B-iii. Proposed Plan of Research

In the coming year we will extend our comparison of the sequences of the mutants obtained from the BB rats from the estrogen-treated and control groups. This is important in confirming that the increased mutagenesis observed in the groups receiving 4-OHE_2 results from DNA damage at AT base pairs. Results from the 4-OHE_2-alone group have not been completed.

The reason for the lack of mutagenicity of the E_2-3,4-Q in BB cells is not known, but the limited stability of this compound may preclude its efficient permeation into the cells. Therefore, we are currently carrying out treatment of the BB cells with an immediate precursor of E_2-3,4-Q, 4-OHE_2. This compound is stable and may be gradually metabolized to E_2-3,4-Q intracellularly. If 4-OHE_2 proves mutagenic, the mutants will be sequenced, and we would expect a mutational specificity similar to that of 4-OHE_2 in BB rats.

We also plan to begin treatments of rats with 4-OHE_2 and the chemopreventive agents, *N*-acetylcysteine with and without vitamins C and E, and 3H-1,2-dithiole-3-thione (D3T). We will also start measuring the mutagenic activity of the estrogenic compounds, estrone, estradiol, estriol, 2-OH-estrone, 4-OH-estrone, and 2-OH-estradiol in BB cells. Dose-response curves and toxicity measurements will be performed to ascertain the optimal dose range for assaying for mutagenesis. The compounds that prove mutagenic in the cell culture assay will then be administered to BB rats, to determine relative mutagenic activity *in vivo*.

C. Key Research Accomplishments

The most significant findings so far, are the observations on abilities of the estrogenic compounds to induce mutagenesis in BB rat breast tissue. A key hypothesis of this proposal was that estrogens have mutagenic effects in addition to their receptor-mediated effects. Also, very significant, is the observation that many of the mutations in rats treated with the 4-OHE_2 + E_2 combination are at A:T base pairs. This was not observed in control rats. As about half of the mutagenesis observed in rats treated with 4-OHE_2 + E_2 results from spontaneous background, and these have only a small percentage of mutations at A:T base-pairs, a high percentage of the 4-OHE_2 + E_2-induced mutations must be attributable to DNA damage at A:T base-pairs.

D. Reportable outcomes

Effects of estradiol, 4-hydroxyestradiol and estradiol 2,3 and 3,4 quinones on mutagenesis *in vivo* and *in vitro*, Wieslawa Kosinska, Zhonglin Zhao, Michael Khmelnitsky, Ercole Cavalieri, Eleanor Rogan, Peter Sacks and Joseph B. Guttenplan
Abstract for Era of Hope Meeting in Philadelphia, June, 2005

E. Conclusions

1. Estradiol, 4-hydroxyestradiol and a combination of the two compounds are mutagenic in BB rat mammary tissue. This is one of the major hypotheses of this project.
2. A substantial portion of the induced mutagenesis by the combination occurs at A:T base-pairs. This observation is consistent with the hypothesis that the estrogen metabolite E_2-3,4-Q is mutagenic and binds to adenines, leading to rapid depurination. Establishing this hypothesis is a major goal of this project.

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SPECIFIC AIM 4 - SANTEN

A. Introduction

Experimental, clinical, and epidemiological data suggest that estrogens contribute to the development of mammary cancer, but the mechanisms responsible remain incompletely understood. The generally accepted hypothesis is that estrogens bind to estrogen receptor (ER) alpha or beta and stimulate the transcription of genes involved in cell proliferation. With each cycle of new DNA synthesis during mitosis, the chances for error increase in DNA replication without adequate repair. As the proliferative process continues, several mutations accumulate. When these mutations disrupt critical regions required for cellular proliferation, DNA repair, angiogenesis, or apoptosis, neoplastic transformation results.

An alternative hypothesis is that estradiol can be converted to genotoxic metabolites and directly damage DNA. The putative genotoxic pathway involves cytochrome P450 1B1 which catalyzes the hydroxylation of estradiol to 4-OH-estradiol. This estrogen metabolite is then further converted to the estradiol 3,4 quinone, which can bind covalently to guanine or adenine, resulting in destabilization of the bond, which links these bases to the DNA backbone. Consequently, adenine and guanine, which are bound to the estradiol-quinone, exit the DNA backbone and form 4-OH-estrone(estradiol)-1-N7-guanine or 4-OH-estrone(estradiol)-1-N3-adenine adducts. With detachment of these two adducts from the DNA backbone, a naked, depurinated site on DNA is left behind. Through the process of error prone DNA repair, these sites now form point mutations which serve as potential initiators of neoplastic transformation. Our working hypothesis is that estradiol acts on both pathways (proliferation and genotoxicity) in a complementary fashion to induce breast cancer.

Our studies, described in detail below, provide further evidence of the validity of the estrogen genotoxic hypothesis. We utilized MCF-7 cells containing a stably transfected aromatase gene and measured genotoxic products after incubation with estrogen substrates. We also used a double transgenic mouse model to dissect out the individual roles of ER alpha and the genotoxic metabolites of estradiol. This animal has the estrogen receptor alpha knocked out (ERKO) and lacks ER beta in mammary tissue. The Wnt-1 transgene is inserted in order to accelerate tumor development. We reasoned that any neoplastic changes induced by estrogens in the ERKO animals must work through estrogen receptor independent pathways.

B. Body

B-i. Methods and Procedures

A full description of the ERKO/Wnt-1 double transgenic animals is included in the original grant proposal. The methods for measurement of the estradiol metabolites were described in the sections written by Cavalieri and Rogan et al and these measurements were made by them as part of the collaboration engendered by this grant. The "estradiol clamp" technique involves use of silastic tubes filled with estradiol diluted in varying ratios with cholesterol. This provides a method to deliver estradiol with zero order kinetics with stability of estradiol levels for up to six months. This method was fully described in the original grant protocol. The aromatase-transfected mice were obtained from Dr. Raj Tekmal and have been fully characterized by him. We have set up our own breeding colony and are generating sufficient mice for all experiments.

B-ii. Results
Specific Aim 4-C

A. Aromatase-transfected MCF-7 cells

We initially determined whether enzymes responsible for formation of depurinating DNA adducts were present in aromatase-transfected human MCF-7 breast cancer cells (1). We detected large amounts of 4-methoxyestradiol, as well as substantial amounts of the quinone conjugates and the depurinated species, 4-OH-estrone(estradiol)-1-N7-guanine in aromatase-transfected MCF-7 cells incubated with 4-OH-estradiol. We then determined whether these cells could aromatize a sufficient amount of testosterone to estradiol to result in formation of the depurinating species. We detected 131 pg/ml of estrogen, indicating the production of estrogens from aromatization. The 4-OH-estrone(estradiol)-1-N7-guanine species was also present at a total concentration (E_1 plus E_2) of 0.17 pg/ml, as were the glutathione, cysteine, and N-acetylcysteine conjugates of estradiol-3,4-quinone. Finally, the aromatase inhibitor, letrozole, inhibited estrogen formation from a total of 131pg/ml of E_1 and E_2 to 2.8 pg/ml and their downstream metabolites to undetectable levels in most cases.

B. Measurements in ERKO/Wnt-1 Mammary Tissue

ERKO/Wnt-1 mammary tissue exhibits an altered metabolic balance (1). Formation of 4-OH-estrogen metabolites are favored over those of the 2-OH species and the catechol-*O*-methyl-transferase pathway appears to be relatively inactive. We detected 10.9 pmol/gm of 4-OHE_2 and 4-OHE_1 in mammary tissue, as well as a total of 2.3 pmol/gm of total conjugated quinone. No 4-methoxyestrogen metabolites were present. These measurements were all made with an assay using HPLC with multi-channel electrochemical detection as the means of detection and quantitation of metabolites.

C. Tumor Incidence in ERKO/Wnt-1 Animals

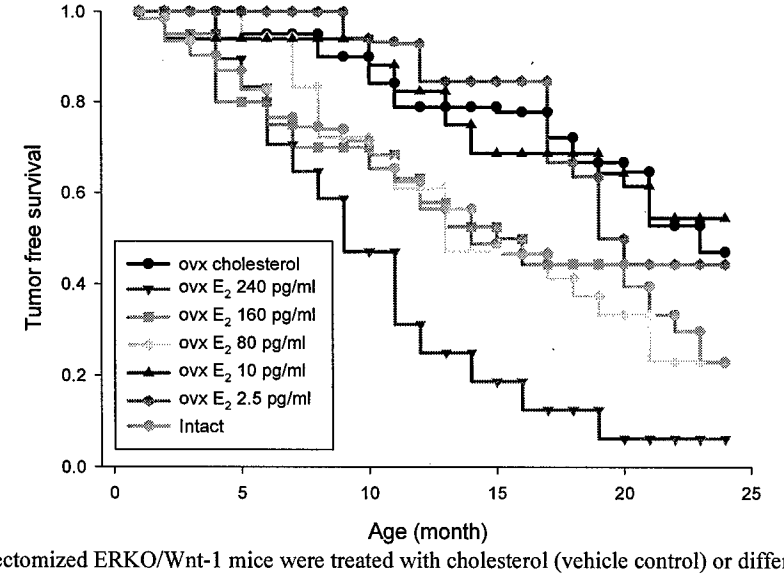


Fig. 1 Intact or ovariectomized ERKO/Wnt-1 mice were treated with cholesterol (vehicle control) or different doses of estradiol delivered by silastic implants. The highest dose of estradiol (240 pg/ml) accelerates mammary tumor formation and increases tumor incidence. The tumor incidences in the mice receiving intermediate doses of estradiol are comparable to that in intact animals. Lower doses of estradiol do not affect tumor formation.

Bocchinfuso et al had previously shown that ERKO/Wnt-1 animals exhibit a delayed onset of tumor development compared to animals expressing the ER alpha. Nonetheless, they observed a nearly 100% incidence of mammary tumors even in the absence of ER alpha and beta. To directly determine the effect of estradiol in the absence of ER, we castrated animals at age 15 days and treated half with silastic implants containing estradiol and the other half with implants of cholesterol. Estradiol was given in increasing doses with the maximal concentration designed to produce plasma levels similar to those seen in intact animals. Implant lengths were designed to "clamp" plasma estradiol at concentrations of 240, 160, 80, 10 and 2.5 pg/ml. After nearly two years of observation, clear dose response effects of estradiol were observed (Fig 1). The highest dose caused tumors to develop at a higher frequency than in the intact animals. In animals receiving the highest dose, more animals developed tumors (15/20 vs 9/20) which appeared earlier than in animals receiving cholesterol implants (time by which 50% of animals developed tumors is 10 months in E_2 group versus 24 months in the control.) ($p<0.004$). The intermediate dose of estradiol (i.e. that producing plasma levels of 80 pg/ml) caused tumors to develop at a slower rate. The lowest doses caused no statistically significant changes in tumor incidence compared to the castrate animals. These data provide evidence that estradiol exerts effects both through an ER alpha dependent pathway, as well as an ER independent pathway to produce breast tumors.

A surprising finding (more fully detailed later in this report) was that the intact animals developed tumors somewhat earlier than did the castrate animals receiving 240 pg/ml of estradiol. This suggests that ovarian components such as progesterone or inhibin might negatively influence tumor formation in the intact animal. Korach and Bocchinfuso previously provided data that supported this concept and suggested that progesterone was a key component. Because of this confounding issue, the comparisons of tumor development in the castrate vehicle control with castrate estradiol replaced animals provides the most precise demonstration of the effects of estradiol.

The ERKO animals used (developed and extensively described by Korach et al) exhibit a residual truncated ER alpha protein which could allow some degree of ER alpha functionality. To examine this possibility we measured uterine weight during long term estradiol administration. As reported by others, the ERKO animals exhibited partial stimulation of uterine growth in response to estradiol (Figure 2). To exclude the effects of minimal ER alpha functionality on tumor development, we treated animals with the pure anti-estrogen, fulvestrant at the same time as estradiol. We reasoned that fulvestrant would down-regulate residual ER alpha activity and also block the transcriptional activity of any remaining. To ensure that the animals received a sufficient amount of fulvestrant to block all estrogen receptor mediated activity, we measured uterine weight as a biologic response parameter. Fulvestrant in the dose used (5 mg/mouse per week, s.c.) caused complete regression of uterine weight to the level observed after castration (Figure 3A). This proved that fulvestrant was abrogating all residual ER alpha or beta activity.

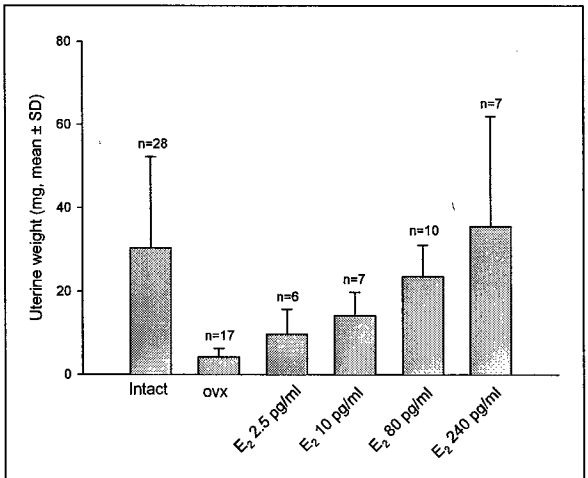


Fig. 2 Uterine weight of ERKO/Wnt-1f intact or ovariectomized mice treated with estradiol.

We then examined whether fulvestrant would inhibit tumor formation induced by estradiol. As shown in Figure 3B, fulvestrant had no effect on tumor formation and estradiol continued to induce tumor formation.

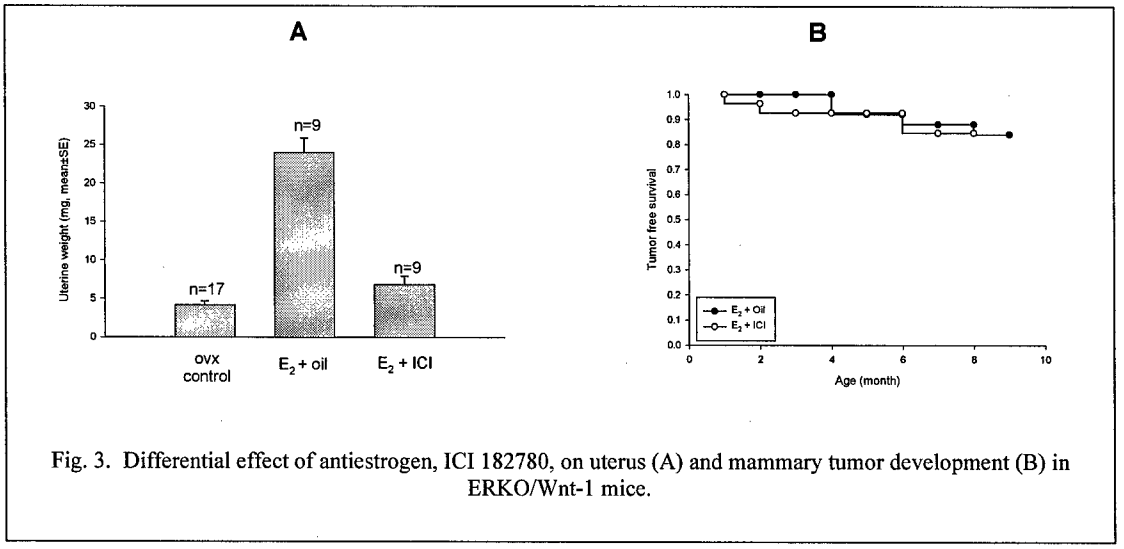


Fig. 3. Differential effect of antiestrogen, ICI 182780, on uterus (A) and mammary tumor development (B) in ERKO/Wnt-1 mice.

Bocchinfuso et al previously reported that products of the ovary could influence mammary tumor formation in ERKO animals. For this reason, we further compared intact with castrate ERKO animals. As mentioned above, of great interest was the observation that intact animals formed tumors more slowly than did castrate animals given estradiol (Figures 1 and 4). Both the intact and the estrogen-treated animals had similar levels of measured estradiol. These data suggest that progesterone or some other ovarian component such as inhibin may be inhibiting tumor formation to some extent. Finally, the ER positive animals develop tumors much more rapidly than either of the ER negative animals, a finding that clearly establishes a role for the ER alpha in the carcinogenic process.

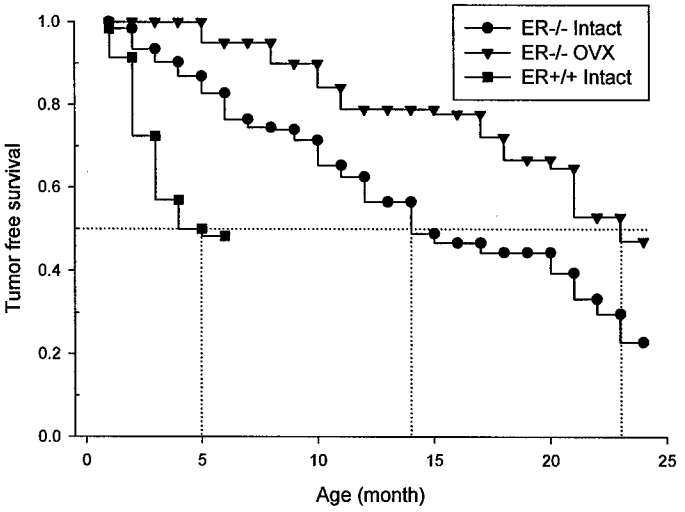


Fig. 4. Mammary tumor formation in Wnt-1 transgenic mice with or without ERα. Time to 50% tumor incidence is about 5 months for ERα positive mice, 14 months for ERα negative mice with intact ovaries, and 23 months for ERα negative castrate mice, respectively.

Specific Aim 4-B.

D. <u>Aromatase-transfected mice</u>

We utilize aromatase-transfected mice to determine the role of genotoxic metabolites of estradiol on tumor formation. In prior studies published by Dr. Raj Tekmal, aromatase-

transfected female mice developed lesions approaching DCIS, as well as atypical ductal hyperplastic lesions. No animals have as yet developed invasive tumors. We are utilizing two strategies to induce tumors in these animals. The first employs the methods of Dr. Dan Medina, who induced tumors by hormonal administration to animals in whom the mammary glands were serially transplanted. Mammary tissue is transplanted into the cleared fat pads of recipient animals. After approximately 24 months of serial transplantation at 3 monthly intervals (8 serial transplants), Dr. Medina frequently observed invasive tumor formation. In this way, he developed three tumor lines in which an insertional mutation upstream of aromatase was associated with tumor formation.

Dr. Ho Yong Park has now been trained to perform this technique by Dr. Raj Tekmal and we have obtained aromatase-transfected mice from him. As shown in Figure 5 we can detect the transgene by PCR and northern analysis. We have begun the serial transplantation process and continue our breeding colony to produce a sufficient number of animals for experimental use. We expect to complete the initial series of serial transplantations in two years. No data are yet available from these studies.

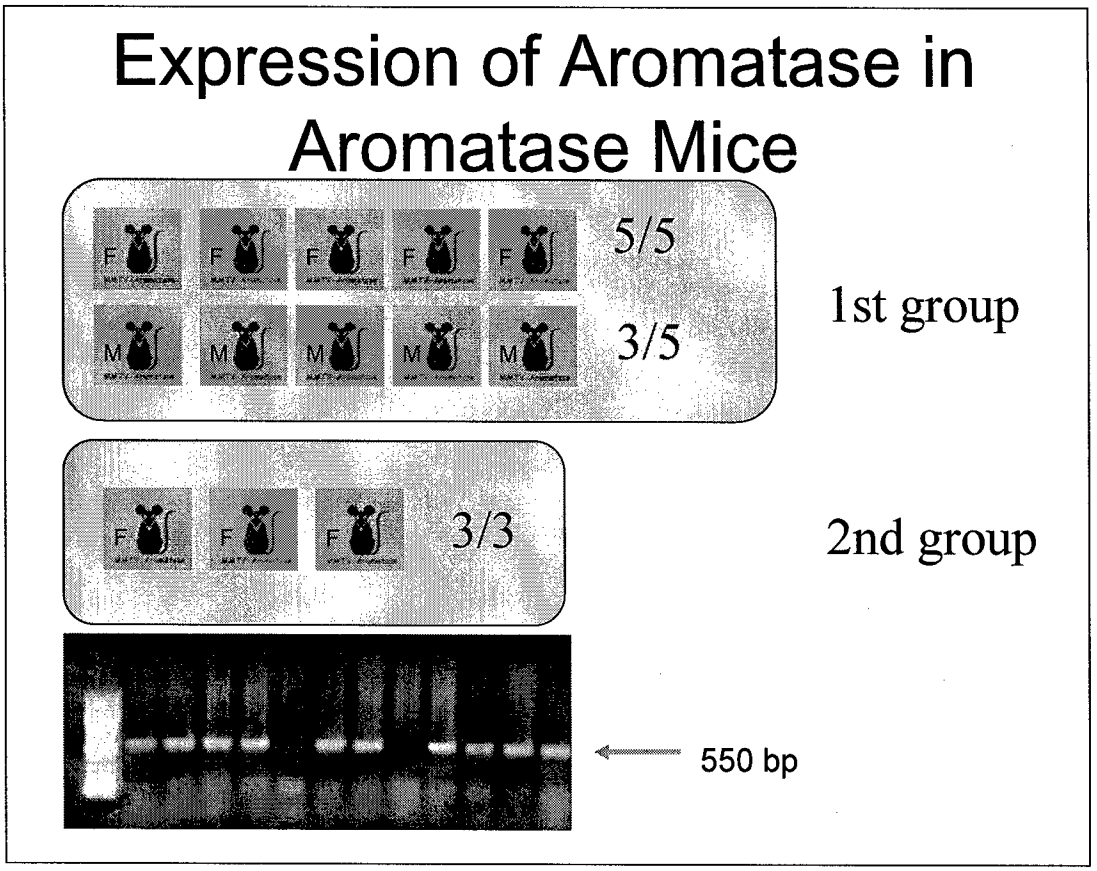


Figure 5. Northern blots demonstrating the aromatase transgene in the aromatase transfected animals.

The second approach is to administer sub-threshold doses of the carcinogen 7,12-dimethylbenzanthracene (DMBA) in a dose response fashion to aromatase-transgenic animals. The concept is that the genotoxic effect of estradiol will interact synergistically with the

subthreshold doses of DMBA. We postulate that this process will be blocked by an aromatase inhibitor as a means of abrogating the substrate for the genotoxic estrogens. These studies have now been started, but no data are yet available.

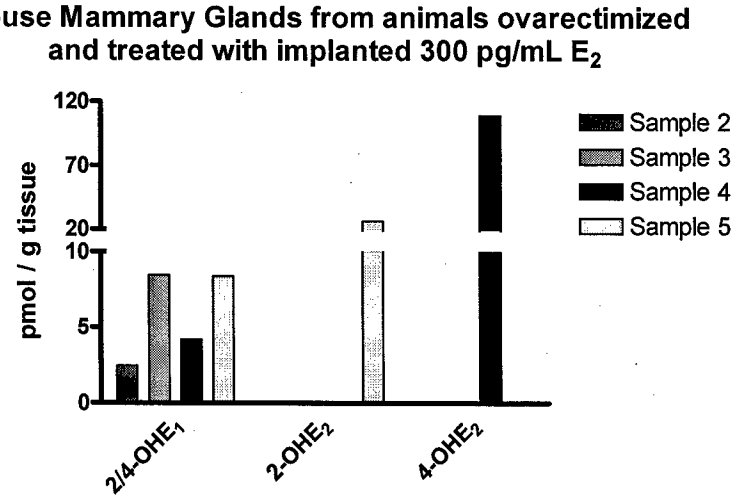
Finally, we have obtained a cytochrome P450 1B1 enzyme inhibitor which blocks the conversion of estradiol to 4-OH-estradiol. This compound is expected to inhibit tumor formation in the aromatase-transgenic animals treated with subthreshold doses of DMBA. These experiments have also been started, but no data are as yet available.

Collaborations:

Collaborations with Drs. Eleanor Rogan and Ercole Cavalieri are ongoing. We are examining three issues. The first is to confirm that the ERKO/Wnt-1 transgenic animals have an imbalance in estradiol metabolism that enhances their ability to form tumors. We previously had shown that the mammary tissue in these animals lacks functional catechol-*O*-methyltransferase based upon the detection of no 4-methoxy estrogen metabolites in benign or malignant breast tissue. Dr. James Yager has directly measured enzymatic activity in these tissues and found it to be normal. Thus we believe that unknown tissue factors must in some way inhibit the activity of this enzyme. We are in the process of examining a larger number of tumors and confirming the results with the newly developed ultra-performance liquid chromatography/tandem mass spectrometry assay.

The second issue is to determine whether the unbalanced estradiol metabolism results from the Wnt-1 transgene, from the knock out of the ER alpha, or from a combination of these two events. Assays of tissues from each of the separate transgenic animals are now ongoing.

Finally, we are measuring the levels of estrone and estradiol and their metabolites in benign breast tissue and in tumor tissue in animals subjected to castration and in castrate animals in whom estradiol has been "clamped" at various levels via silastic implants. These measurements were first performed using the HPLC-electrochemical detector method, but this technique seems not to have sufficient specificity for tissue measurements. We are now using the UPLC/mass spectrometry technique to assess the levels in tumor tissue. Our preliminary results are illustrated in Figure 6 below.



B-iii. Proposed Plan of Research

During the next grant year we will administer 17-alpha estradiol to the ERKO/Wnt 1 animals to determine if an estrogen that can be poorly converted to genotoxic metabolites will influence the development of tumors. This experiment is to provide further evidence that genotoxic metabolites in the absence of ER alpha or beta can contribute to cancer development. These experiments will utilize the same methodology as utilized for the experiments illustrated in Figure 1. In the next year, we will serially transplant the aromatase-transfected mammary glands into cleared mammary pads according to the method of Medina et al. We will continue the experiments to administer sub-carcinogenic amounts of dimethylbenzanthracene to the aromatase-transfected animals. These studies will involve administration of the aromatase substrate androstenedione with and without the aromatase inhibitor letrozole. In this way, we will be able to demonstrate a key role for the aromatase enzyme in the process of carcinogenesis. The animals to be used for these experiments have now been generated in our breeding colony and the experiments are ongoing.

C. Key Research Accomplishments

The major accomplishment to date is to provide a proof of principle demonstration that estradiol, independently of its effect mediated through the estrogen receptor, can accelerate and enhance the development of breast cancer. This finding has substantial clinical significance for the prevention of breast cancer in women. If the genotoxic and estrogen receptor mediated effects of estradiol are complementary, as suggested by the data, aromatase inhibitors will be much more effective in the prevention of breast cancer in women than tamoxifen. The aromatase inhibitors block both the ER mediated and the genotoxic pathways, whereas tamoxifen only blocks ER mediated effects. It should be noted that 5 studies in women (ATAC, IES, MAP 17, Big Fempta and ABC/ARNO) have shown that aromatase inhibitors are more effective in preventing contralateral breast cancer in the adjuvant setting than is tamoxifen. These clinical data are best explained by the hypothesis of additive or synergistic effects of estradiol on the process of mammary carcinogenesis.

The second key observation is that mammary tissue contains the enzymes necessary to convert androgens to genotoxic estradiol metabolites. The theory that genotoxic metabolites can cause breast cancer requires that breast tissue contain these metabolites. Our HPLC and mass spectrometry measurements have shown that mammary tissue from our experimental systems contain the necessary enzymes. These data come from the aromatase-transfected MCF-7 cells, as well as from measurements in the mammary tissue of the ERKO/Wnt-1 animals.

Finally, our data support the concepts of Bocchinfuso and Korach that the ovary produces substances that can negatively influence breast cancer development in rodents. We have shown that castrate animals given estradiol develop mammary tumors earlier than do intact animals with the same levels of estradiol. These observations suggest that either progesterone, inhibin, or other compounds made by the ovary could mediate these effects.

D. Reportable Outcomes

The reportable outcomes are documented in the list of publications provided in the reference section below. In summary, we report the rate of development of breast tumors in the ERKO/Wnt-1 animals under various physiological circumstances and the levels of genotoxic metabolites in the mammary glands of these animals or in MCF-7 cells. We are planning in the near future to report in a publication all of the data from the ERKO/Wnt-1 animals. This

information will be presented orally at three upcoming plenary sessions, one at the Annual Meeting of the Endocrine Society to an audience of approximately 5000 attendees, the second at the American Clinical Ligand Society in San Antonio on May 14 and the third at the DoD Era of Hope meeting in Philadelphia in June.

E. Conclusions

A variety of evidence suggests that estrogens can contribute to the production of breast cancer. The commonly held theory is that estrogens stimulate cell proliferation, increase the number of genetic mutations in proportion to the number of mitotic divisions, and promote the propagation of these mutations by stimulating growth. Our alternate hypothesis suggests that estrogens may be metabolized to directly genotoxic compounds. Our working construct is that these two pathways act in concert in an additive or synergistic fashion to cause breast cancer. In support of this hypothesis, we demonstrated that MCF-7 cells can convert 4-OH-estradiol and testosterone both to the 4-OH-estrone(estradiol)-1-N7-guanine depurinating adduct and to estrogen quinones. We also demonstrated that the incidence of breast tumor development in ER-alpha knock out (ERKO) transgenic animals could be enhanced by administration of estradiol to oophorectomized animals. These new data provide direct new evidence of the biological importance of the genotoxic pathway.

Our first aim was to demonstrate that human breast cancer cells convert testosterone or 4-OH-estradiol to genotoxic products. We clearly demonstrated this in an MCF-7 cell model system by using a highly sensitive and specific assay for steroid measurements. A commonly expressed criticism of the genotoxic hypothesis is that supra-physiologic amounts of estrogen are needed to form genotoxic metabolites of estradiol. Our *in vitro* experiments can be criticized on the same basis. However, we believe that biologic endpoints of this process provide a higher level of sensitivity than do biochemical measurements. This reasoning is supported by studies which examined the biologic effects of estrogen under similar *in vitro* conditions. Russo et al, our collaborator in this Center of Excellence, have shown that 0.007 nM estradiol can induce neoplastic transformation, as evidenced by increased colony formation in benign MCF-10F cells which lack a functional ER. Similar concentrations induce loss of heteozygosity (LOH) in benign, non-ER breast cells at hot spots for LOH in breast cancer tissue. Such low concentrations can also induce mutations in V-79 cells. As further evidence of the ability of physiologic amounts of estrogen to serve as substrate for these genotoxic metabolites, human breast cancer tissue and surrounding benign breast contain large amounts of these metabolites. Taken together, the tissue measurement data and the findings from incubated cells *in vitro* clearly demonstrate that human breast tissue can form substantial amounts of the genotoxic metabolites of estradiol.

The ERKO/Wnt-1 animals provide a powerful model for studying the effect of estrogen in the absence of a functioning ER. These animals have circulating estradiol levels in the range of 325 pg/ml (K. Korach, personal communication, 2002). This is approximately 30-50 fold higher than normal as a consequence of the absence of estradiol negative feedback on the pituitary and the resultant rise in luteinizing hormone (LH) levels. In addition, the breast tissue from these animals appears to convert little 4-OH-estradiol to 4-methoxy-estradiol, a metabolite which is thought to be inactive and to obviate further conversion to genotoxic metabolites.

We consider it highly relevant with respect to prevention of breast cancer to determine whether or not the genotoxic pathway is biologically important. Anti-estrogens act only to block

ER mediated function, whereas the aromatase inhibitors reduce estradiol levels and consequently block both ER mediated as well as genotoxic pathways. Theoretically, aromatase inhibitors would then be much more efficacious for prevention of breast cancer than the anti-estrogens. Data from the recently reported ATAC trial, as well as from the IES, MAP 17, Big FEMPTA, and ABC/ARNO trials, can be interpreted in light of the genotoxic hypothesis. In these trials, the aromatase inhibitors resulted in a 50% greater reduction of invasive contralateral breast cancer than did the anti-estrogen, tamoxifen. While there are other explanations for these differences, the magnitude of the greater effect of the aromatase inhibitor is substantial. These observations, when taken together with the biologic data presented in this report, highlight the compelling need to determine conclusively whether or not the genotoxic hypothesis of estradiol-induced carcinogenesis is operative.

The ERKO animals used in this study continue to express a truncated form of the ER alpha. This may be capable of mediating estradiol effects via non-genomic mechanisms. There may also be some residual ER beta in breast tissue which is not detected with current techniques. In order to address these theoretic issues, we have administered fulvestrant to the ERKO/Wnt-1 animals to completely block and down regulate residual estrogen receptors. These animals continue to produce breast tumors, indicating that tumors form even with the combined strategies of transgenic ER knock out and use of pure antiestrogens to block estrogen receptor mediated functions.

F. References

Published Manuscripts:

1. Yue, W., Santen, R.J., Wang, J-P., Li, Y., Verderame, M.F., Bocchinfuso, W.P., Korach, K.S., Devanesan, P., Todorovic, R., Rogan, E.G., and Cavalieri, E.L. Genotoxic metabolites of estradiol in breast: potential mechanism of estradiol induced carcinogenesis. Journal of Steroid Biochemistry and Molecular Biology 86:477-486, 2003.

2. Santen, R.J. Inhibition of aromatase: insights from recent studies. Steroids 68:559-567. 2003.

3. Yue, W., Wang, J-P., Bocchinfuso, W., Korach, K., Devanesan, P., Rogan, E., Cavalieri, E., and Santen, R.J. Tamoxifen versus aromatase inhibitors for breast cancer prevention. Clinical Cancer Research 11:925s-930s, 2005.

Peer Reviewed Chapters:

4. Santen, R.J., Yue, W., Bocchinfuso, W., Korach, K., Wang, J.-P., Rogan, E., Li, Y., Cavalieri, E., Russo, J., Devanesan, P., and Verderame, M. Estradiol-induced carcinogenesis via formation of genotoxic metabolites, IN: Advances in Ednocrine Therapy of Breast Cancer: Proceedings of the 2003 Gleneagles Conference, Ingle, J.N. and Dowsett, M. (eds), Marcel Dekker, New York, NY, pp. 13-177, 2004.

Abstracts:

5. Santen, R.J. Endocrine Society Plenary Session: "Development of aromatase inhibitors for the treatment and prevention of breast cancer" 87th Annual meeting of the Endocrine Society, San Diego California, June 4-7, 2005.

6. Santen, R.J. American Clinical Ligand Society Distinguished Scientist Award Lecture: Role and growth factors and estrogen metabolism in the growth and development of breast cancer. San Diego, California, May 12-14, 2005.

SPECIFIC AIM 5 - INGLE

Work on Specific Aim 5 has begun with the start of the third year of the Center, as proposed.

MOLECULAR BIOLOGY CORE – SUTTER

A. Introduction

Approved Workstatement for Year 2

Perform microarray analyses and RealTime PCR measurements on samples from animals and cell culture.

The molecular biology core of this BCCOE brings cutting edge molecular analyses to the specific aims of this Center. The primary capacity of this core is high throughput gene expression analysis facilitated by Affymetrix GeneChip technology. In addition to massively parallel analysis of gene expression, new technologies, released in 2005, permitted the expansion of these facilities into high resolution analysis of chromosome structure. As determined at the December 6, 2004 meeting of the BCCOE held in Washington, DC, the efforts of this core in Year 2 focused on **Specific Aim 2 (Russo)** of the Center: **to determine the effects of estrogen and its metabolites on the progressive steps of neoplastic transformation of human breast epithelial cells (HBECs) and to determine whether the neoplastic phenotypes and genotypes thus induced can be abrogated by known and new preventive agents.**

B. Body

B-i. Methods and Procedures

Following on the successful transformation of the spontaneously immortalized human breast epithelial MCF-10F cells by treatment with estrogens, reported by Dr. Russo in year 1, the Russo and Sutter labs began an active collaboration to define the molecular events that occur during the progressive transformation of MCF-10F cells. Genomic DNA and total RNA were extracted from independent cell isolates of the following cell lines and tissues: **1)** The parental, immortal but not transformed, cell line MCF-10F, MCF-10F-1, -2 and -3; **2)** The 70 nM E_2-transformed cells of MCF-10F, E_2-70 nM-1,-2, and -3; **3)** The clone C5 of the 70 nM E_2-transformed cells, selected after invasive growth through a Boyden Chamber, C5-70 nM-1, -2, and -3; **4)** Three of the six sub-clones of clone C5 of the 70 nM E_2-transformed cells; C5-Clone-A, C5-Clone-B, and C5-Clone-F; **5)** The tissue of the four tumors produced in SCID mice by the injection of C5 clone cells, Tumor-An-1, -4, -5, -6; **6)** The human tumor cell lines established from the tumors produced in SCID mice by injection of C5 clone cells, C5-tumor-1, C5-tumor-2, and C5-tumor-8.

Genomic DNA was isolated using a Proteinase K, phenol extraction, ethanol precipitation method and total RNA was isolated using the Trizol method. The quality of DNA and RNA was determined spectrophotometrically and by capillary gel electrophoresis. Affymetrix 100k SNP mapping was performed using the combined Xba I and Hind III 50 k SNP GeneChip Mapping Array set according the manufacturer's recommended procedures (Affymetrix). Gene expression microarray analysis was performed using the newest complete human genome transcript chip, the Human Genome U133 Plus 2.0 Array, which simultaneously measures more than 47,000 transcripts (Affymetrix).

The Affymetrix GeneChip Mapping set uses two arrays to determine the allele call of more that 100,000 single nucleotide polymorphisms. Applications of this technology include dense whole genome scans, analysis of loss of heterozygosity (LOH), and case-control genome association studies. Using the 100k SNP set, the investigator obtains allele information at a

mean intermarker distance of 22.5 kb and a median intermarker distance of 8 kb. The average heterozygosity of each SNP is 0.29. An overview of this method is shown in **Figure 1.**

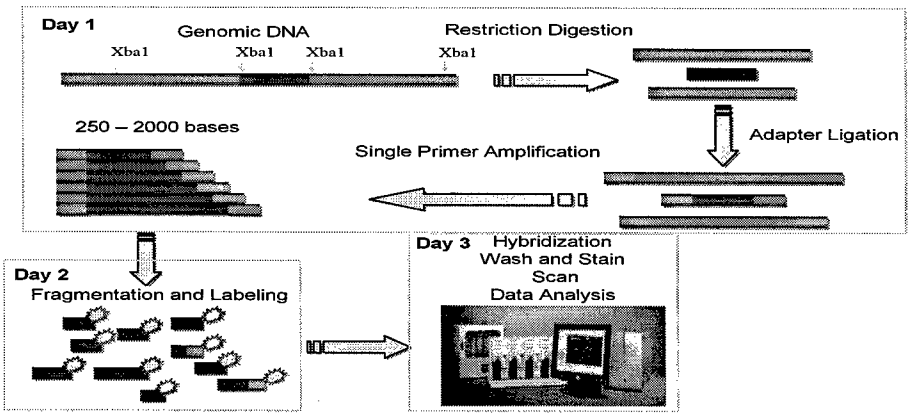


Figure 1. Single Primer Assay Overview. A PCR-based method is used to reduce genomic complexity by amplifying regions of DNA that are near informative SNPs. All of the sites were chosen using bioinformatics and analysis of 3 million SNPs from the Perlegen and public databases. Basically, the protocol is to digest the DNA with a restriction endonuclease, add linkers, amplify smaller and specific regions of genomic DNA using a single primer PCR reaction, fragment and label the DNA and hybridize to a SNP microarray chip. After analysis, each SNP is assigned a call of AA, AB or BB.

B-ii. Results

In the initial study, we have used the 100k SNP GeneChip Mapping Array set to interrogate the structure of chromosomes 1 – 22 and X at very high resolution. The results of this analysis are shown in **Figure 2.** Relative to cell transformation, the MCF-10F and the E_2 transformed cells are more similar, as are the Boyden chamber selected clone, its subclones and the animal tumor cells. Allelic imbalances that reflect these groupings are found on chromosomes 4, 8 and 13. LOH on chromosomes 4 and 8 is associated with chromosome deletion while the LOH observed on chromosome 13 is not. We also see three regions specifically associated with cells from tumors in animals on chromosomes 3, 9 and 18.

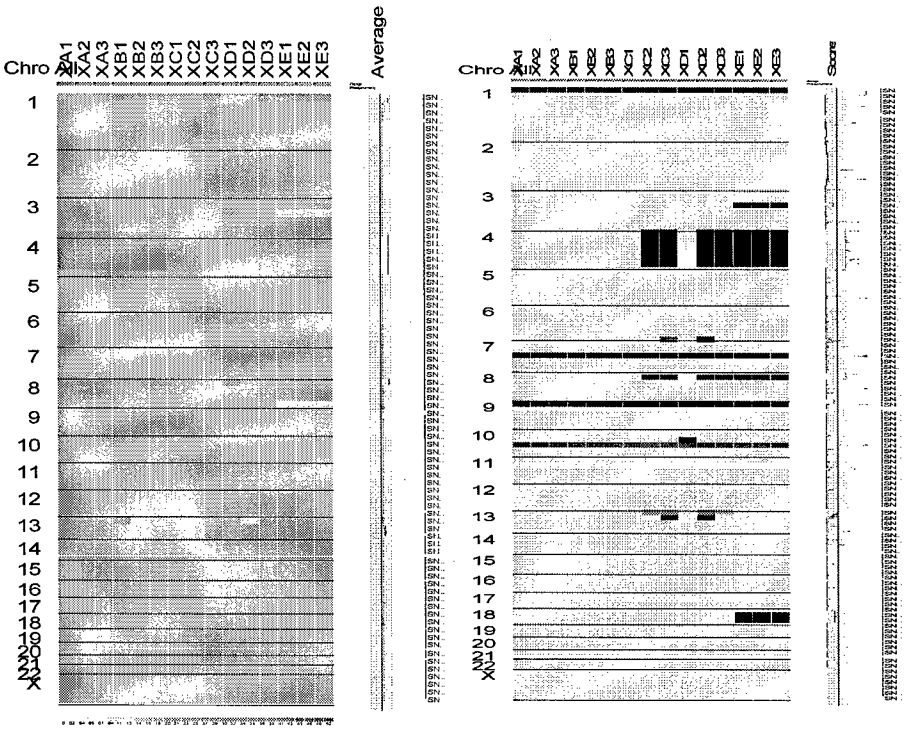


Figure 2. Concurrent analysis of chromosomal copy number (CN) and loss of heterozygosity (LOH). dCHIP software (Chen Li, Harvard University, updated for current annotation by Dr. Huang of the Sutter lab) was used to examine both copy number and allelic alterations. Left Panel (Pink). Complete genome view of copy number, with darker and lighter areas representing areas of copy number differences among the 15 samples. Right Panel (Yellow/Blue). Complete genome view of LOH. **XA1-3)** The parental, immortal but not transformed, cell line MCF-10F, MCF-10F-1, -2 and -3; **XB1-3)** The 70 nM E2-transformed cells of MCF-10F, E2-70 nM-1,-2, and -3; **XC1-3)** The clone C5 of the 70 nM E2-transformed cells, selected after invasive growth through a Boyden Chamber, C5-70nM-1, -2, and -3; **XD1-3)** Three of the six sub-clones of clone C5 of the 70 nM E2-transformed cells; C5-Clone-A, C5-Clone-B, and C5-Clone-F; **XE1-3)** The human tumor cell lines established from the tumors produced in SCID mice by injection of C5 clone cells, C5-tumor-1, C5-tumor-2, and C5-tumor-8.

It should be noted that the samples corresponding to the tissue of the four tumors produced in SCID mice by the injection of C5 clone cells, Tumor-An-1, -4, -5, -6, could not be analyzed using the GeneChip Mapping Array. We believe that this is due to cross-contamination of the human breast tissue with mouse DNA from infiltrating cells, *in vivo*. Fortunately, Dr. Russo also isolated cell lines from these tumors (samples XE1-3, below), which have been shown to provide important new information about tumorigenesis *in vivo*.

As major structural alterations were observed for chromosome 4, we present this chromosome at higher resolution in **Figure 3.** Of particular interest to this analysis, sample XD1 (Sub-clone of clone C5, C5-Clone A) shows more limited regions of LOH than do the other samples analyzed. As Dr. Russo has recently shown that this sub-clone produced tumors when injected into SCID mice (unpublished results), it appears likely that analysis of specific sub-clone data, such as this example, will narrow the possible candidate genes for progression of cell transformation/breast cancer. Further high resolution analysis of this data will identify specific genes that are likely to be involved in tumorigenesis (for example, see **Figure 4**).

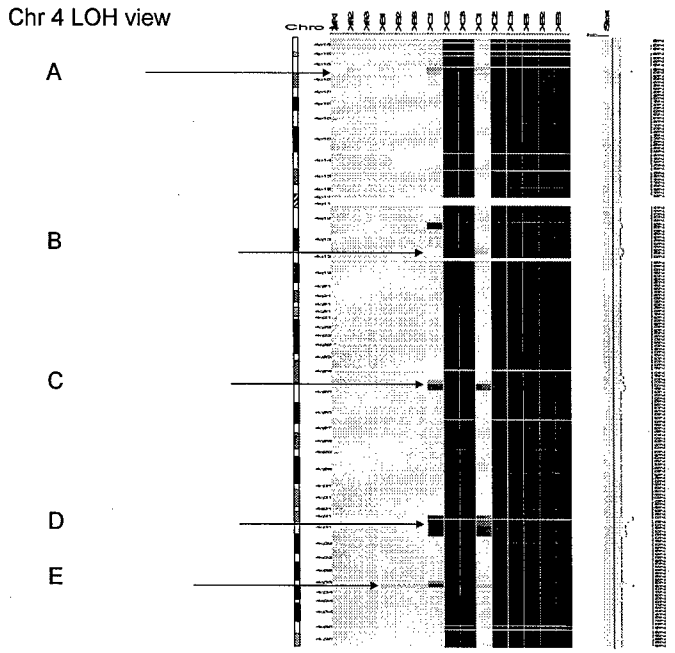


Figure 3. Higher resolution of chromosome 4 LOH. Of interest, XC1 (C5 sample) and XD1 (clone A of C5) show much restricted regions of LOH. As these samples also produce tumors in animals, this analysis indicates that subclones can be used to establish finer maps of potential candidate genes. These five regions of interest are identified by arrows A-E.

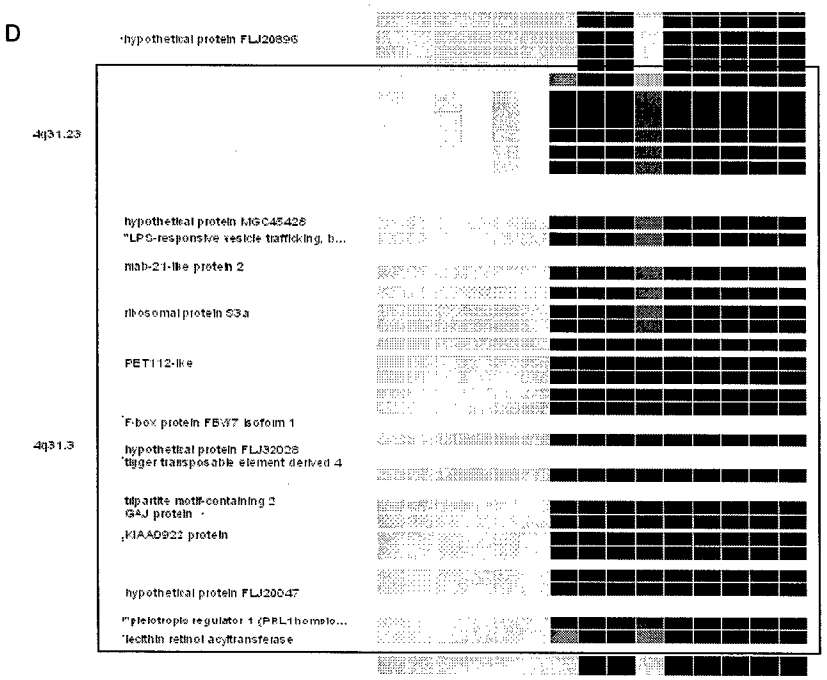


Figure 4. High resolution LOH analysis of region D of chromosome 4. Region D of chromosome 4 identified by an arrow in Figure 3 is shown in greater detail. At this level of resolution, individual genes, potential breast cancer genes, can be identified by gene ID and name.

B-iii. Proposed Plan of Research

In year 3, we will continue the analysis of progression of breast cell transformation in collaboration with Dr. Russo (Specific Aim 2). In the first publication, we will describe the alterations in chromosome structure identified above. In a second manuscript, we will combine chromosome structure analysis with RNA microarray analysis in order to i) further narrow the list of breast cancer candidate genes and ii) to identify pathways and gene networks involved in cell transformation, *in vitro*, and breast cancer development, *in vivo*.

C. Key Research Accomplishments

Using a well characterized *in vitro-in vivo* model of breast carcinogenesis, we have demonstrated the ability to characterize cell transformation at the combined levels of the complete genome and the individual gene. This research should lead to vastly improved understandings of cell transformation and tumorigenesis and permit translational studies to human breast cancer cases.

D. Reportable Outcomes

See Russo report for abstract presented at the 2005 annual AACR meeting. This work will also be presented at the upcoming Era of Hope meeting and the Gordon conference on hormonal carcinogenesis.

E. Conclusions

We have established a strong and functional collaboration within the BCCOE. Initial results demonstrate the progression of cell transformation leading to tumorigenesis *in vivo*, and demonstrate our ability to move effectively between studies of chromosome structure and gene function.

F. References

1. Russo, J., Lareef, MH, Tahin, Q, Hu, YF, Slater, C, Ao, X and Russo, IH (2002) 17β-estradiol is carcinogenic in human epithelial cells. J. Steroid Biochem. Mol. Biol. 80: 149-62.

2. Fernandez, SV, Russo, IH, Lareef, M, Balsara, B and Russo, J (2005) Comparative genomic hybridization of human breast epithelial cells transformed by estrogen and its metabolites. Int. J. Oncol. 26: 691-5.

3. Lin, M, Wei, LJ, Sellers, WR, Lieberfarb, M, Wong, WH and Li C (2004) dChipSNP: significance curve and clustering of SNP-array-based loss-of-heterozygosity data. Bioinformatics 20: 1233-1240.

ANALYTICAL CORE – ROGAN

A. Introduction

The Analytical Core provides consistent analytical power to the research projects so that estrogen metabolites, estrogen conjugates and estrogen-DNA adducts can be identified and quantified with the most sensitivity and reliability. The HPLC with multi-channel electrochemical detection enables detection of 31 metabolites and conjugates at the picomole level in one run. A second set-up is used exclusively to analyze the 6 depurinating catechol estrogen-DNA adducts, which need a different elution buffer to achieve separation. Our newer ultra-performance liquid chromatography/tandem mass spectrometry (UPLC/MS/MS) instrumentation has increased the sensitivity of our analyses to the femtomole level and provides confirmation of structures.

B. Body

B-i. Methods and Procedures

The Analytical Core uses both HPLC with electrochemical detection and UPLC/MS/MS to analyze estrogen metabolites, conjugates and depurinating DNA adducts. A variety of types of samples are analyzed. These include *in vitro* reaction mixtures, human and animal tissue extracts, cell culture medium extracts, and human nipple aspirate fluid. Depending on the sample, preparation for analysis is as simple as filtration through a 5,000-molecular weight filter, or as complex as grinding minced tissue in liquid nitrogen, incubation with glucuronidase/sulfatase, passage through a Sep-Pak column to extract desired analytes, and filtration through a 5,000-molecular weight column.

B-ii. Results

In the past year we concluded that capillary HPLC was not able to resolve the key estrogen metabolites and GSH conjugates of interest in our LC/MS/MS analyses. Therefore, we purchased new technology, the Acquity ultra-performance liquid chromatography (UPLC) from Waters, Inc. This has enabled us to reduce the time required for each sample from 1 h to 15 min. More importantly, we can now separate and analyze all of the estrogen metabolites, GSH conjugates [and their breakdown products containing cysteine (Cys) and *N*-acetylcysteine (NAcCys)] and depurinating DNA adducts by UPLC/MS/MS except 2-hydroxyestrone (2-OHE_1) and 4-OHE_1. We are continuing to resolve this one remaining problem.

We have analyzed a few nipple aspirate samples with this technology:

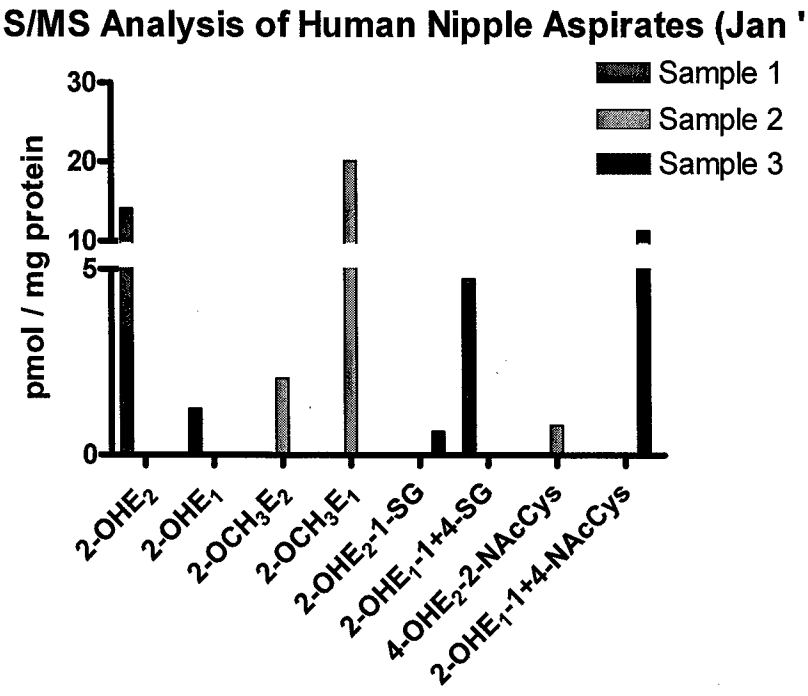


Figure 1: UPLC analysis of nipple aspirate fluid.

In these samples we detected 2-OHE_2 and 2-OHE_1, the methylated catechol estrogens, 2-OCH_3E_1 and 2-OCH_3E_2, the GSH conjugates 2-OHE_2-1-SG and 2-OHE_1-(1+4)-SG, as well as the breakdown products 2-OHE_1-(1+4)-NAcCys. The only detected compound coming from the 4-catechol estrogens was 4-OHE_2-2-NAcCys. We are continuing to refine these analyses. We are not surprised to find primarily analytes containing the 2-catechol estrogens because these samples all came from healthy women without breast cancer. In women with breast cancer we would expect to see higher levels of the 4-catechol estrogens and their derivatives [1].

We also found that when the nipple aspirate samples are passed through a 5,000-molecular weight filter before UPLC/MS/MS analysis, the filter can be extracted to yield RNA for analysis by the Molecular Biology Core.

We have analyzed several samples of medium from MCF-10F cells treated with E_2 or 4-OHE_2 in Specific Aim 2 (Russo). These results are not ready to report. We hope to have good results for Dr. Russo to report at the Era of Hope meeting in June.

We have begun analyzing mouse mammary tissues from ERKO/Wnt-1 mice that were ovariectomized and then implanted with E_2 or cholesterol in Specific Aim 4 (Santen). We are still working out some sample preparation conditions, including the optimal amount of tissue to use and whether to treat with glucuronidase/sulfatase before extracting the tissue.

B-iii. Proposed Research for the Next Year

1. Analyze nipple aspirate fluid samples from women with and without breast cancer (Specific Aim 1).

2. Begin to provide RNA from nipple aspirate samples to the Molecular Biology Core for analysis of enzyme expression.

3. Continue to analyze media from MCF-10F cells treated in various ways (Specific Aim 2).

4. Continue to analyze mammary tissue from ERKO/Wnt-1 mice treated in various ways (Specific Aim 4).

C. Key Research Accomplishments

We have set up the new Acquity UPLC system with our MS/MS and resolved all of our 31 estrogen metabolites, GSH conjugates and depurinating adducts, except for the 2-OHE_1 and 4-OHE_1.

We have successfully analyzed human nipple aspirate fluid and detected estrogen metabolites and GSH conjugates.

D. Reportable Outcomes

Rogan, E.G. and Cavalieri, E.L. Estrogen metabolites, conjugates and DNA adducts: Possible biomarkers for risk of breast, prostate and other human cancers, In: Adv. Clin. Chem., (G.S. Makowski, G. Nowacki & K.-J. Hsiaom Eds.), 38:135-149, 2004.

Rogan, E.G. and Cavalieri, E.L. The role of estrogen metabolism in the initiation of human breast cancer. Era of Hope, Department of Defense Breast Cancer Research Program Meeting, Philadelphia, Pennsylvania, June 8-11, 2005.

E. Conclusions

The Analytical Core has successfully implemented UPLC/MS/MS to detect estrogen metabolites and GSH conjugates in human nipple aspirate fluid from healthy women without breast cancer. Refinements in techniques can be anticipated that will enable us to detect depurinating DNA adducts, which would be expected to be present at higher levels in women with breast cancer [1].

The Analytical Core is interacting with the other investigators in the Breast Cancer Center of Excellence to analyze a variety of samples for estrogen metabolites, estrogen GSH conjugates and depurinating estrogen-DNA adducts.

F. References

1. Rogan, E.G., Badawi, A. F., Devanesan, P.D., Meza, J.L., Edney, J.A., West, W.W., Higginbotham, S.M., and Cavalieri, E.L. Relative imbalances in estrogen metabolism and conjugation in breast tissue of women with carcinoma: Potential biomarkers of susceptibility to cancer. *Carcinogenesis*, 24: 697-702, 2003.

ADVOCACY CORE – HART

Web-based model reference tool

Our web-based model reference tool of basic scientific terminology has progressed to the second iteration. We were unable to solve the glitch in Microsoft Word and therefore determined that another avenue had to be found. We wanted to be able to screen any scientific journal article prior to its appearance on a web page and have the terms defined automatically as one's cursor passed over the word. We tried downloading medical dictionaries, which made it easier to access word definitions, but there was no mechanism or software application to screen a scientific journal article and automatically assign definitions to scientific words. Having discussed our idea with multiple information systems' personnel and software developers, we finally were put in contact with a company that develops software for CompUSA. TraCorp, Inc. suggested that scientific journal articles appear as web pages in a normal, web browser window, and before the web server sends the page, it automatically runs an application that adds invisible hyperlinks to each word it matches to a medical term in its dictionary. Thus, when reading a journal article, a user can roll the mouse over dictionary words and automatically pop up the definition. We discussed this solution with the DoD grant administrator assigned to our grant, hoping that the Army with its extraordinary software capabilities might be willing to take the idea and develop it for use on its DoD Congressionally Directed Medical Research and Materiel Command's web page, particularly in screening scientific journal articles that were produced by DoD funded research. The grant administrator felt that this was not the purview of their department, but would run it through channels and suggested that we write an additional grant to develop the tool, as we do not have sufficient funding in the present grant to develop this further. Undaunted, we are planning to approach TraCorp and ask if they would be willing to donate their services to help us bring our idea to fruition. When this is complete, we will follow our original plan to test its usefulness and then disseminate as originally discussed.

Consumer Guide to Involvement in Basic Research

The Consumer Guide, *Advocates and Scientists, Partners in Research,* is nearing completion and will be ready for review by selected advocates in the next two-three months, as well as other members of the Center of Excellence Grant.

Article for publication based on current research

We have not begun this project as yet, but expect to do so in the next few months.

We continue to meet with the other members of the Center of Excellence Grant and contribute to discussions from the consumer perspective.